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University
of Glasgow

BACTERIAL FUNGAL INTERACTIONS IN THE CYSTIC FIBROSIS LUNG

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**A Thesis submitted to the University of Glasgow for the
Degree of Masters of Science (by Research)**

In

Glasgow Dental School,
School of Medicine,
College of Medical, Veterinary and Life Sciences

Submitted May 2013

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Abstract

Pseudomonas aeruginosa is the major bacterial pathogen found in Cystic Fibrosis (CF) patients, with up to 80% being colonised by the age of 18. Within the CF lung, however, polymicrobial infections are increasingly common. In fact, the saprophytic fungi *Aspergillus fumigatus* is increasingly observed in association with *P. aeruginosa*, which is correlated with poorer clinical outcomes. The aim of this project was therefore to investigate whether the interaction between *P. aeruginosa* and *A. fumigatus* resulted in changes to its virulence potential using a range of *in vitro* and *in vivo* experimental methodologies.

The series of experiments described herein investigated the effect that *A. fumigatus* biofilms had on the induction of a surrogate virulence factor elastase from *P. aeruginosa* through the use of a biochemical assay. Elastase is produced by many strains of *P. aeruginosa* and causes host damage by cleaving elastin within the CF lung. *A. fumigatus* biofilms were grown in a 24-well plate format and live and ethanol-killed biofilms of various stages of development were produced (8 h germlings, 12 h monolayer of proliferating mycelia, 24 h and 48 h mature biofilm). These were then exposed to planktonic wild type and clinical strains of *P. aeruginosa*. Dead *A. fumigatus* biofilm was shown to increase elastase significantly in multiple strains, including a mucoid strain. It was also shown that the intact biofilm structure was essential to this increase in elastase, as disrupting the dead *A. fumigatus* biofilm and treating *P. aeruginosa* with the lysate did not result in a significant increase in elastase. Interestingly, the effect was restricted to dead biofilms, as presence of live *A. fumigatus* biofilm resulted in a significant decrease in elastase produced by certain *P. aeruginosa* strains.

Despite a significant change in expressed elastase being found for *P. aeruginosa* type strain PA01 at the phenotypic level, a real time qPCR approach did not show any significant up-regulation of *LasB*, the gene encoding elastase, over multiple time points in response to dead 24 h *A. fumigatus*. The possibilities of transient gene expression or mRNA degradation with an increase in protein expression are potential explanations for this.

Cell culture studies utilising the A549 human lung epithelial cell line aimed to determine the effects of both supernatants from combinational cultures and elastase itself on a cell line of relevance to the CF lung. Elastase was shown to detach A549 cells from the plastic surface they were grown on and adherent to using light microscopy, with no loss of viability as measured by alamarBlue. The effect of combinational *P. aeruginosa*/*A. fumigatus* supernatants in contrast with their respective controls were difficult to determine due to the highly cytotoxic effect of live *A. fumigatus* in this project.

A *Galleria mellonella* wax worm insect model was also utilised to test for synergy in *P. aeruginosa*/*A. fumigatus* co-infections. This was performed by establishing the effect of an optimised *P. aeruginosa* inoculum on wax worm survival treated with dead *A. fumigatus* 8 h germlings. No significant difference were shown in the survival rates of this model when worms were injected with combinations of *P. aeruginosa* and killed *A. fumigatus* 8 hour germlings, though melanisation of the worms indicated an additive effect on the organisms.

In conclusion, these studies demonstrated a relationship between *A. fumigatus* biofilms and an increased virulence expression from *P. aeruginosa* using a surrogate virulence marker, elastase. Due to its known negative effects in regards to human health, this suggests that polymicrobial infections of both *P. aeruginosa* and *A. fumigatus* could generate increased morbidity in those susceptible to these infections. Future work should be undertaken to unravel the exact mechanisms behind increased virulence expression in *P. aeruginosa* in response to *A. fumigatus*, and determine fully whether this is of important clinical significance and therefore a key bacterial/fungal interaction to focus on within CF studies.

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Acknowledgement

Throughout my time at Glasgow Dental School and Hospital, I was fortunate to receive invaluable help and guidance during my Masters project, and I would like to take this opportunity to acknowledge the following people:

This research was made possible by generous funding from the NHS, for which I am eternally grateful. I would like to thank Prof. Gordon Ramage in particular for giving me the opportunity, and for his continual support, encouragement and advice both in regards to my studies and my career. In addition to this, Dr. David Lappin was a great asset around the lab and in helping to shape the overall project, which is much appreciated.

Special thanks are due to Dr. Ranjith Rajendran for his key role in introducing me to postgraduate laboratory work, and sharing his wide and detailed knowledge in the field of mycology and bacteriology with me.

My time in the laboratory was also greatly enhanced through support by the other postgraduate students working on Level 9 at the Dental School, although Miss Leighann Sherry and Miss Emma Millhouse in particular deserve a mention for their expertise with *in vitro* studies, and cell culture work, respectively. The warm and welcoming environment in the Dental School (and outside of it!) made for a very memorable year which I look back on fondly, and I hope to keep in touch with the many interesting characters I met there.

Outside of the Dental School, I would like to acknowledge my parents, grandparents, brother and friends for their encouragement, even when they weren't quite sure why I was going to the lab to look at wax worms at 1 in the morning.

Declaration of Originality

I am aware of and understand the University's policy on plagiarism and I certify that this thesis is my own work, except where indicated by referencing.



Stephen Kerr, May 2013

Definitions

3OC12HSL	3-oxo-C12-homoserine lactone
A549	Human lung adenocarcinoma epithelial cell line
ABPA	Allergic broncho-pulmonary aspergilliosis
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATTC	American type culture collection
BHI	Brain Heart Infusion
cDNA	Complimentary deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator protein
cfu	Colony forming unit
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's media
DMSO	Dimethylsulfoxide
ECR	Elastin-Congo Red
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
h	Hour
<i>hip</i>	High persistence mutants
IgE	Immunoglobulin E
IL	Interleukin
kDa	Kilo Dalton
<i>LasB</i>	Elastase structural gene
<i>LasI</i>	Autoinducer synthesis protein <i>LasI</i>
<i>LasR</i>	Transcriptional regulator <i>LasR</i>
LB	Luria broth
LDH	Lactate dehydrogenase
M	Mucoid
min	Minutes
mm	Millimetre
µg	Microgram
µl	Microlitre
mg	Milligram
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
N	Non-mucoid
NAD	Nicotinamide adenine dinucleotide
NDK	Nucleoside diphosphate kinase
nm	Nanometer
O.D.	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	<i>Pseudomonas</i> Elastase
<i>proC</i>	Pyrroline-5-carboxylate reductase
qPCR	Real time PCR
REDOX	Reduction-oxidation

RNA	Ribonucleic acid
RNA-Seq	Whole Transcriptome Shotgun Sequencing
rpm	Rotations per minute
RPMI-1640	Roswell Park Memorial Institute-1640
<i>rpoD</i>	RNA polymerase, sigma 70 (sigma D) factor
SAB	Saboraud Dextrose Agar
SEM	Scanning electron microscopy
TE buffer	Tris-EDTA buffer
Th2	T helper cell type 2
Tris-HCl	Trizma hydrochloride

1 Introduction

1.1. General Introduction

Since Alexander Fleming discovered the antibiotic penicillin, a result of a serendipitous observation of a contaminated agar plate on which interactions between staphylococci and the then-unknown mould *Penicillium* spp. were observed, it has been evident that there are important consequences of such interactions (Ligon 2004). Indeed, given the abundance of bacteria and fungi in the environment, which can infect humans, these interactions are probably common and therefore have potentially important implications for health. Despite this, research into the interactions between fungi and bacteria both *in vitro* and *in vivo* is scant. We have yet to discover in full the consequences of these interactions, the mechanisms involved, and how they impact the host.

1.2. Cystic Fibrosis

With an incidence of 1 in 2500 of new-borns in the UK, Cystic Fibrosis (CF) is the most common lethal genetic disorder within the Caucasian population, affecting over 7000 people in the United Kingdom (Davies *et al.*, 2007). CF is a recessive autosomal genetic disorder which leads to abnormally viscous mucus within the respiratory system, generally resulting in early mortality due to an opportunistic infection of the respiratory tract. These are caused by bacterial pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Proesmans *et al.*, 2008) and fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* (de Vrankrijker *et al.*, 2011).

In the 1950's, this genetic disorder meant that death by 5 years of age was probable. Fortunately, since then diagnosis and treatment methods have improved, meaning the prognosis is considerably more optimistic today, with mean survival of 50 years and over being achieved (Dodge *et al.*, 2007). However, early mortality and morbidity is still observed within CF patients so further progress is essential. In particular, studies into the impact of the bacteria and fungi which co-infect the CF respiratory tract is one area of particular interest. As little data exists about how these interactions impact

upon the health of CF patients, novel treatment methods and understanding of the disease pathogenesis could be uncovered (Hogan. 2009).

1.2.1. Genetic basis for CF

CF is caused by a mutation in the gene on chromosome 7 which encodes for cystic fibrosis transmembrane conductance regulator (CFTR) protein (Bobadilla *et al.*, 2002). There are many different mutations of CFTR that can lead to CF, with in excess of 1500 mutations being implicated in the disease (O'Sullivan and Freedman 2009). Although there is much work to be done in understanding a number of these, the functional alterations of some of the most common mutations have been detailed. Five different classes have been described which correspond to the various functional alterations resulting from the gene mutation, as detailed in Table 1.1 (Proesmans *et al.*, 2008; Treacy *et al.*, 2011).

Table 1.1. Mutation classes leading to CFTR protein dysfunction in Cystic Fibrosis patients.
Adapted from Proesmans et al., 2008 and Treacy et al., 2011.

Class	Mutation	Molecular consequence
I	Nonsense G542X Frameshift 394delTT Splice junction 1717-1G→A	No CFTR messenger ribonucleic acid resulting in no CFTR protein formed
II	Missense AA deletion $\Delta F508$	Trafficking defect due to aberrantly folded protein which is degraded by the cell quality control mechanism, resulting in little to no CFTR protein present in the cell.
III	Missense G551D	Full length CFTR protein is synthesised and incorporated into the cell membrane, but the regulation is defective leading to no function.
IV	Missense R117H	Defective chloride conductance.
V	Missense A455E Alternative splicing 3849+10kbC→T	Deregulation of transcription due to a splicing defect leads to a decreased amount of normal CFTR.
VI	Q1412X	Unstable CFTR protein produced, causing an increase in ions other than chloride through the apical channels.

A class II mutation in which the phenylalanine at position 508 is deleted, accounts for around two thirds of the North American and North European CF population (O'Sullivan and Freedman 2009). This results in a protein trafficking defect in which ubiquitination of the protein occurs and CFTR protein is degraded by the endoplasmic reticulum or Golgi body (Farinha and Amaral 2005).

The CFTR protein is expressed at the apical borders of epithelial cells in the exocrine glands throughout the body and has several important functions including an essential role in ion and fluid homeostasis (Riordan 2008). CFTR acts predominantly as a chloride channel (Sheppard and Welsh 1999), but also has other regulatory roles such as ATP channel regulation, inhibition of calcium-activated chloride channels and bicarbonate-chloride exchange. The most commonly accepted theory for why defects in CFTR result in disease is the "low volume" hypothesis (Treacy *et al.*, 2011). This states that the dysfunctional CFTR fails to prevent sodium absorption through the sodium epithelial channel leading to hyper-absorption of water, and a depletion of the surface airway liquid. Due to this, the substantial mucus which is secreted into airways becomes more viscous as it becomes dehydrated and thus more difficult to clear from the respiratory tract. In effect, mucociliary cleaning of the respiratory tract becomes less effective. This provides an environment in which opportunistic microbes can colonize the airways. The presence of these pathogens within the ordinarily sterile lung leads to inflammation of the respiratory tract, which results in damage to the tissues of the lungs. Over time, the function of the CF lung decreases due to this damage, correlating with disease progression in the patient (O'Sullivan and Freedman 2009). The most significant of the bacterial pathogens impacting upon CF patients is *Pseudomonas aeruginosa*. As such, this organism is of key interest to research into CF.

1.3. *Pseudomonas aeruginosa* and its role in cystic fibrosis

P. aeruginosa is a ubiquitous Gram-negative rod-shaped bacterium which is found in soil, water supplies and the skin microflora, thus creating a high risk of exposure for CF sufferers. *P. aeruginosa* has been found within water supplies in hospital environments, affecting showers, ice makers, flower vases and even within some medications such as eye drops (Kerr and Snelling 2009). For example, in January 2012 three premature babies died of *Pseudomonas* infection in the Royal Jubilee Maternity Hospital in Belfast, whilst three others became infected (one dying from other causes, the other two recovering). *P. aeruginosa* was also detected on the skin of five other babies who did not become infected (Wise 2012). Although it is of low virulence within an uncompromised population, *P. aeruginosa* is an opportunistic pathogen that can colonise various parts of the body such as the eyes, burn wounds or in the case of CF patients, the lungs (Lyczak *et al.*, 2000). In fact, *P. aeruginosa* ranks within the top five organisms responsible for pulmonary, bloodstream, urinary tract, surgery site and soft tissue infections within intensive care units (Trautmann *et al.*, 2005).

Hosts with a competent immune system effectively clear *P. aeruginosa* from the respiratory tract via mucociliary action and innate immune mechanisms (Sadikot *et al.*, 2005). However, immunocompromised hosts, such as AIDS patients, and those suffering from genetic disorders (CF), are at a distinct disadvantage in preventing airway colonisation by *P. aeruginosa* (Dropulic *et al.*, 1995).

Exposure of CF patients to *P. aeruginosa* is considerable, and by the age of 18 up to 80% of patients with CF will have been colonized by this organism, showing *P. aeruginosa* is a key pathogen for research within this area (San Gabriel *et al.*, 2004).

1.3.1. *Pseudomonas* biofilms in the CF lung

In order to establish a chronic infection in the host, *P. aeruginosa* forms biofilms within the CF lung. This was confirmed by the demonstration that the quorum sensing signals measured in CF sputum samples were consistent with that of biofilm being present, as opposed to planktonic, cells. Moreover, microscopy of the CF sputum revealed that *Pseudomonas* was present in biofilm-like structures (Singh *et al.*, 2000).

Biofilms are communities of microbes, be it one or multiple species, encased in an extracellular matrix composed of polysaccharide, proteins, glycolipids, extracellular DNA and cellular debris (Branda *et al.*, 2005). The matrix generally makes up 90% of the biofilm dry mass (Flemming *et al.*, 2007). In terms of human infection, including those within the CF lung, the species within these communities can include fungi and bacteria. Biofilms are attached to a surface, generally in contact with some form of aqueous fluid. They are complex three-dimensional structures, containing water channels which allow necessary materials such as nutrients to be moved throughout the biofilm community. These structures are also variable and dependent on conditions, such as the availability of nutrients (Hall-Stoodley *et al.*, 2004). The process of biofilm formation is detailed in Figure 1.1.

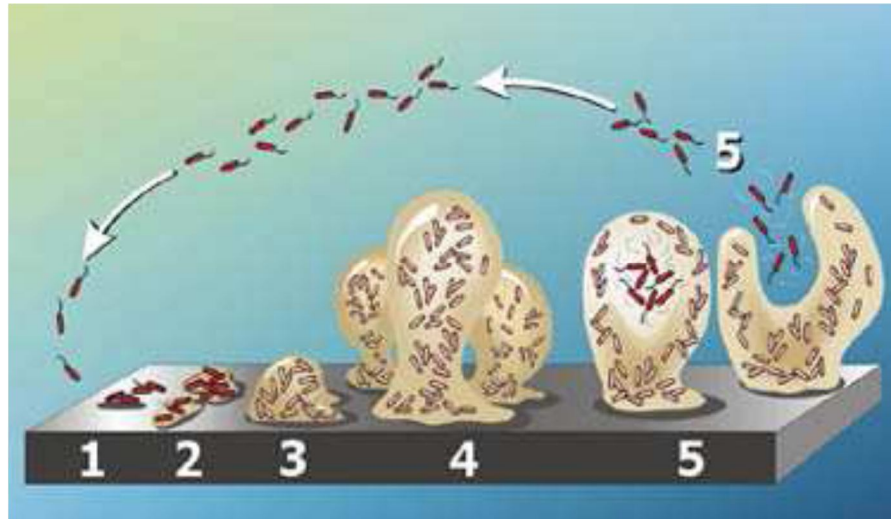


Figure 1.1. Summary of the basic steps in bacterial biofilm formation.

1. Initial reversible attachment of planktonic microbes to a surface by weak interactions such as Van der Waal's forces. 2. Irreversible attachment of microbes to the surface, associated with a phenotypic change in the organisms. This single layer of microbes begins slime production, releasing the extracellular matrix. 3. This single slime layer begins vertical development, producing a more three dimensional structure. 4. The maturing biofilm, by which stage "towers" of biofilm are present containing water channels to facilitate nutrient acquisition by microbes within the inner layers. 5. The mature biofilm. At this stage, free-floating planktonic bacteria disperse from the biofilm and colonise other areas and initiate further biofilm formation (Watnick and Kolter 2000). Taken from Dr. David G. Davies Lab, Birmingham University (<http://www2.birmingham.ac.uk/biology/faculty/davies/research.htm>)

The mature *P. aeruginosa* biofilm can generally never be cleared from the CF lung. The persistence of *P. aeruginosa* as a biofilm within the CF lung has been linked to multiple factors including a decreased production of antibacterial nitric oxide in CF patients (Chmiel and Davis 2003). Moreover, *P. aeruginosa* biofilms are mostly drug-resistant making treatment especially difficult (Drenkard, 2003). The secretion of viscous mucus within the CF lung leads to a low oxygen environment which is thought to contribute to the growth of *P. aeruginosa* on top of the lung epithelial cells (Moreau-Marquis *et al.*, 2008).

Upon initial infection by a *P. aeruginosa* clonal strain, the clone commonly persists for multiple years within the airway, diversifying due to mutations and the composition of its accessory genome. Multiple clonal strains can co-colonise within the same host (Cramer *et al.*, 2010). Excessive inflammatory reaction initiated by the biofilm is likely to be connected to the hosts' inability to eradicate the infection. Therefore, host damage occurs, with IL-8 and leukotriene B4 production causing the recruitment of neutrophils, which release mediators such as neutrophil elastase (Radake *et al.*, 2011). Antibiotic treatment is largely unsuccessful even though the *P. aeruginosa* within the lungs are not necessarily antibiotic-resistant (El Solh and Alhajhusain 2009).

Persister cells are known to occur within the *P. aeruginosa* populations within the CF lungs as high persistence (*hip*) mutants (Mulcahy *et al.*, 2010). It has been hypothesized that persister cells evade antibiotic treatment and allow drug-resistance mutants to develop and persist within the lungs (Horre *et al.*, 2010). Persister cells are dormant variant cells which arise sporadically within microbial populations, such as the biofilm and are metabolically inert. As antibiotics function on active targets, the dormant persister cells evade killing and can repopulate later. These cells contribute heavily to relapse in those with biofilm infections, as shown in Figure 1.2 (Lewis 2010).

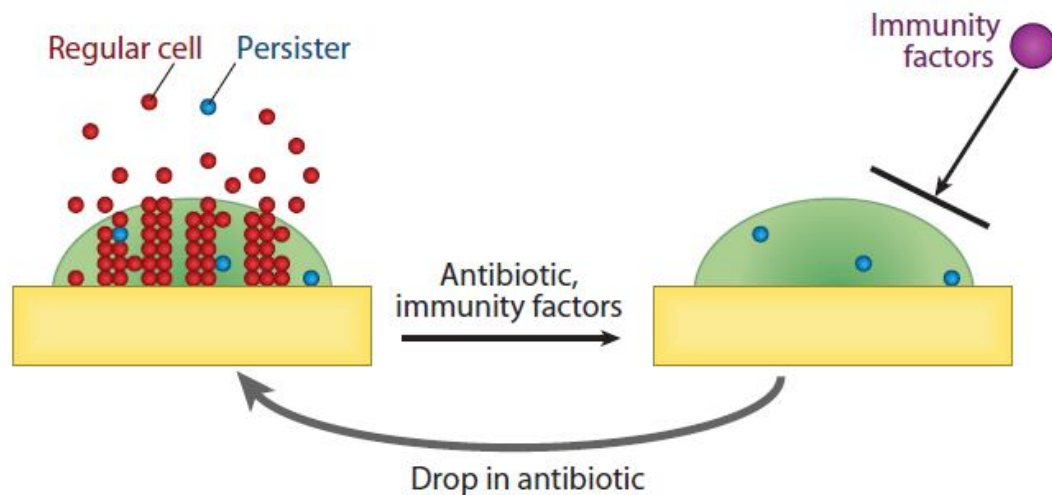


Figure 1.2. Both regular and persister cells form within the biofilm.

Within a host, both regular cells and persister cells shed from the biofilm into the bloodstream, with antibiotics killing only the regular cells in the bloodstream and the immune system responsible for clearing the persister cells. Antibiotic therapy also kills the regular cells within the biofilm, but the persister cells remain in their dormant state, protected by the matrix of the biofilm. When the concentration of antibiotics drops, the persister cells in the biofilm become active again and repopulate the biofilm, leading to recurring infection (Lewis 2010).

1.3.2. *Pseudomonas aeruginosa* virulence factors

P. aeruginosa is associated with various virulence factors, and these include pseudomonad pyocyanin, rhamnolipid, alkaline protease, superoxide dismutase and elastase (pseudolysin), a key factor in pathogenicity (Mulcahy *et al.*, 2010). The expression of these virulence factors varies over the course of infection due to microevolution, with the bacteria remaining virulent throughout the course of the CF patient's life (Bragonzi *et al.*, 2009).

1.3.2.1. Elastase as a virulence factor

Pseudomonas elastase (PE), also known as pseudolysin, is one of the most damage-inducing extracellular virulence factors acting on the host. Secretion of this virulence factor has been detected in over 75% of *P. aeruginosa* clinical isolates (Kuang *et al.*, 2011). Encoded by the *lasB* gene, it is a 39.5-kDa zinc metalloproteinase which degrades the elastin present in the human lung, whilst

also acting on other matrix proteins such as laminin and collagen types III and IV (Adekoya and Sylte 2009). It is a member of the thermolysin family (M4) of proteins, of which many members are virulence factors produced by pathogenic microorganisms including λ -Toxin from *Clostridium perfringens* (Adekoya and Sylte 2009).

PE has been shown to be involved in the pathogenicity of pseudomonads in the host via three different mechanisms (Hoge R. 2010): (1) Direct tissue damage by PE leads to improved growth conditions for bacteria due to an increase in nutrient concentration, leading to an increased bacterial load which can contribute to further damage. (2) Further activity of PE can degrade various plasma proteins, including immunoglobulins, complement factors and cytokines. (3) There is also the possibility that an increased inflammatory response will occur due to the recruitment of neutrophils to the respiratory tract, producing a destructive inflammatory cycle (Kon *et al.*, 1999; Schmidtchen *et al.*, 2003).

Therefore, the risk of continued infection is increased due to PE as it lowers the defence mechanisms of the lung and destroys important surfactant proteins. The inflammatory response can also be triggered by the presence of a biofilm, which PE also has a role in by activating nucleoside diphosphate kinase (NDK) within bacterial cells, an important enzyme which is key to cellular macromolecular synthesis and signalling and therefore has an important role in bacterial growth, signal transduction and pathogenicity (Chakrabarty 1998; Cathcart *et al.*, 2011).

Host cells are also capable of producing their own elastase. Within the context of airway pathogenicity, neutrophil elastase is the most significant form of the enzyme, being found at substantial concentrations in the surface airway fluids of those suffering from airway diseases (Fischer and Voynow 2002). With elastase being produced by some host cells themselves and by multiple pathogens within the CF lung, the chances of significant damage being caused is increased.

Elastase has been implicated in causing chronic ulcers due to the degradation of host skin proteins and wound fluids (Schmidtchen *et al.*, 2003), as well as causing corneal liquidation (Willcox 2007). The presence of elastase in the lung has been shown to lead to structural damage by degrading basal membranes and

the tight junctions of epithelia. For example the presence of desmosine, a by-product of elastin breakdown in the lungs that can be detected in the urine to indicate elastolytic activity against elastin, has been shown to be elevated in CF patients (Viglio *et al.*, 2000). It has also been demonstrated in animal models that elastase-deficient strains are less virulent within the lungs, and that the host is also better able to clear the bacterial infection (Blackwood *et al.*, 1983). Due to ease of colonisation by *P. aeruginosa* of CF patients, and therefore exposure of their lungs to elastase, this is of particular interest in CF studies.

The scenario is complicated enough with a single species infection of the CF lung, but in reality the ecology of the CF lung has been shown to be polymicrobial, and the interactions between these different microorganisms can be hypothesised to lead to a dynamic change in the expression of virulence factors, and could possibly contribute further to the difficulty in eradicating the biofilm via synergy between species. This is part of the rationale behind studies into bacterial-fungal interactions within disease.

1.4. *Aspergillus fumigatus* and its role in cystic fibrosis

In addition to bacterial pathogens, yeasts such as *C. albicans* (Latge 1999) and filamentous fungi such as *A. fumigatus* (Gomez *et al.*, 2010) also colonise the CF respiratory tract.

A. fumigatus is a saprophytic fungus, living on dead organic matter, with its natural environmental niche being within the soil. *A. fumigatus* is ubiquitous due to a high yield of airborne spores which are released as conidia into the atmosphere, explaining the high rates of exposure in CF patients (de Vrankrijker *et al.*, 2011). It has been found that on average we inhale at least several hundred conidia each day with some estimates claiming that 1 to 100 colony forming units of *A. fumigatus* can be found for each cubic meter of indoor and outdoor air (Knutsen and Slavin 2011). The conidia are small enough (2-3µm) to become located in the alveoli of the lung following inhalation. *A. fumigatus* is the most prominent filamentous fungi which infect CF patients, with prevalence

rates ranging from 6% to 58% depending on the study (Burns *et al.*, 1999). Other species of *Aspergillus* are also associated with infection, including *A. flavus* and *A. niger*, but *A. fumigatus* is by far the most prominent species in human infection.

A. fumigatus produces biofilms in a different way from *P. aeruginosa* and other bacterial and fungal species, as shown in Figure 1.3 (Ramage *et al.*, 2011).

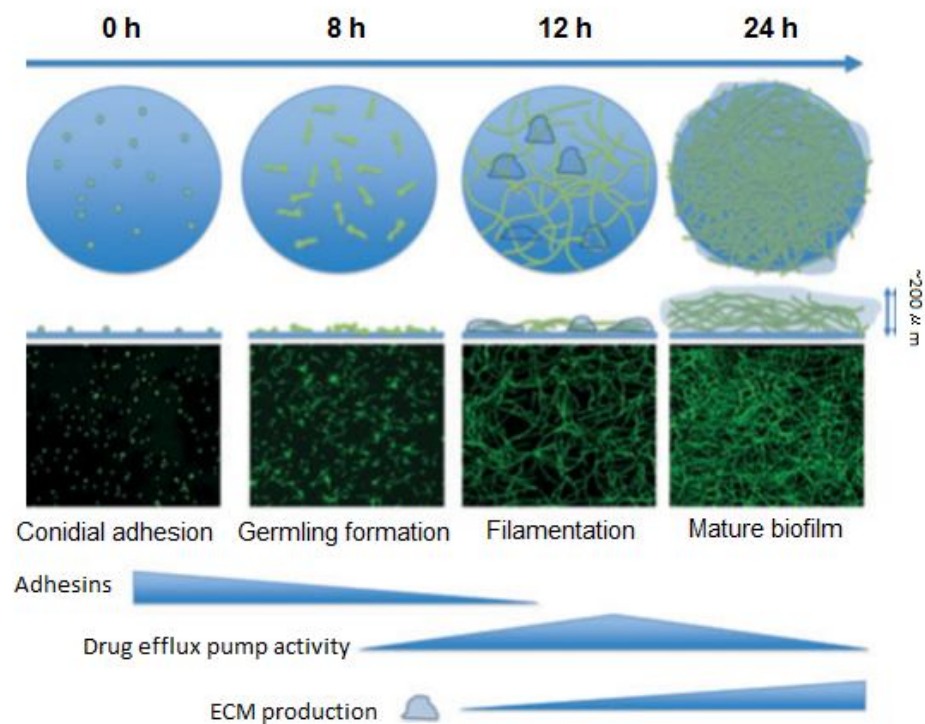


Figure 1.3. Biofilm formation of *Aspergillus fumigatus*.

Biofilm development begins with initial adhesion of a conidial spore to a surface (0 h), before germling formation (8 h), development of a monolayer of intertwined hyphae (12 h) and finally a mature 3D filamentous biofilm mass (approximately 200 μm) encased with an extracellular matrix (24 h). Confocal laser scanning micrographs stained using FUN1 cell viability stain demonstrate the appearance of *A. fumigatus* within the biofilm at each respective stage of development. Three key gene classes associated with biofilm (adhesins, drug efflux pump activity and extracellular matrix) are indicated with their optimum time of expression. Taken from Ramage *et al.*, 2011.

A. fumigatus is also capable of producing elastase. This was shown to be related to the pathogenesis of the fungi via an animal model (Kothary *et al.*, 1984). In this model, 6 elastase-producing and 4 non-elastase producing strains were tested in immunocompromised mice. It was found that the elastase positive strains caused death in all mice tested, with hyphal growth and alveoli necrosis occurring within the lungs. The elastase negative strains did not lead to destruction of the alveoli, and few hyphae were found in the lung tissue. This indicates that elastase production may be important to *A. fumigatus* colonisation and invasion as with *P. aeruginosa*.

The most common disease caused by *A. fumigatus* in CF patients is allergic broncho-pulmonary aspergilliosis (ABPA) which affects 7-9% of CF patients (Knutsen and Slavin 2011). Those suffering from asthma are also at particular risk, with 1-2% of asthmatics suffering from ABPA (Luzzaro *et al.*, 2011). ABPA develops due to T helper cell type 2 (Th2) hyper-sensitivity to *A. fumigatus* hyphae colonizing the respiratory tract. It is characterized by pulmonary infiltrates, with the lungs becoming filled with fluid. An *A. fumigatus*-specific immune response via IgE and IgG is noted along with an increase in IgE levels. *A. fumigatus* is also associated with asthma, bronchitis and aspergilloma.

One study by Burns *et al.* on 520 patients showed that there was an increase in detected levels of *A. fumigatus* and *C. albicans* after six months of inhaled tobramycin treatment being utilised against *P. aeruginosa* against a control group (Burns *et al.*, 1999). It is still to be determined whether or not antibiotic therapy simply allows colonisation by fungi, or whether it changes the environment of the CF lung allowing for the existing fungi to grow more prominently. It does raise questions as to whether treatments against bacteria within the CF lung could be beneficial for fungi, and vice versa.

1.5. Mixed Bacterial and Fungal Interactions in the CF Lung

The presence of bacteria and fungi within the CF lung has been known about for some time, yet looking at the interactions between such organisms in order to determine whether this leads to an increase in pathogenicity has been somewhat neglected. These studies could also lead to the discovery of mechanisms that microbes use to compete in a polymicrobial environment, which could ultimately be exploited in order to find novel treatments.

Polymicrobial infections have been shown to occur fairly often in patients. For example, one Italian report based on data gathered from April 2007 over multiple diagnostic centres indicated that approximately 8.5% of bloodstream infections were polymicrobial (Frey-Klett *et al.*, 2011). Another clinical study of 94 patients with CF with a median age of 28 by Bakare *et al.*, in 2003 showed that *A. fumigatus* was present in 45.7% of sputum samples and of these samples 64.2% were also detected to have *P. aeruginosa* co-infection. Figure 1.4 shows a summary of reported co-infections of microbial species within the CF lung.

It has also been shown that these types of infections are more likely to lead to a less favourable disease outcome. Therefore, interactions between species such as *A. fumigatus* and *P. aeruginosa* are liable to occur within the context of CF and should be studied.

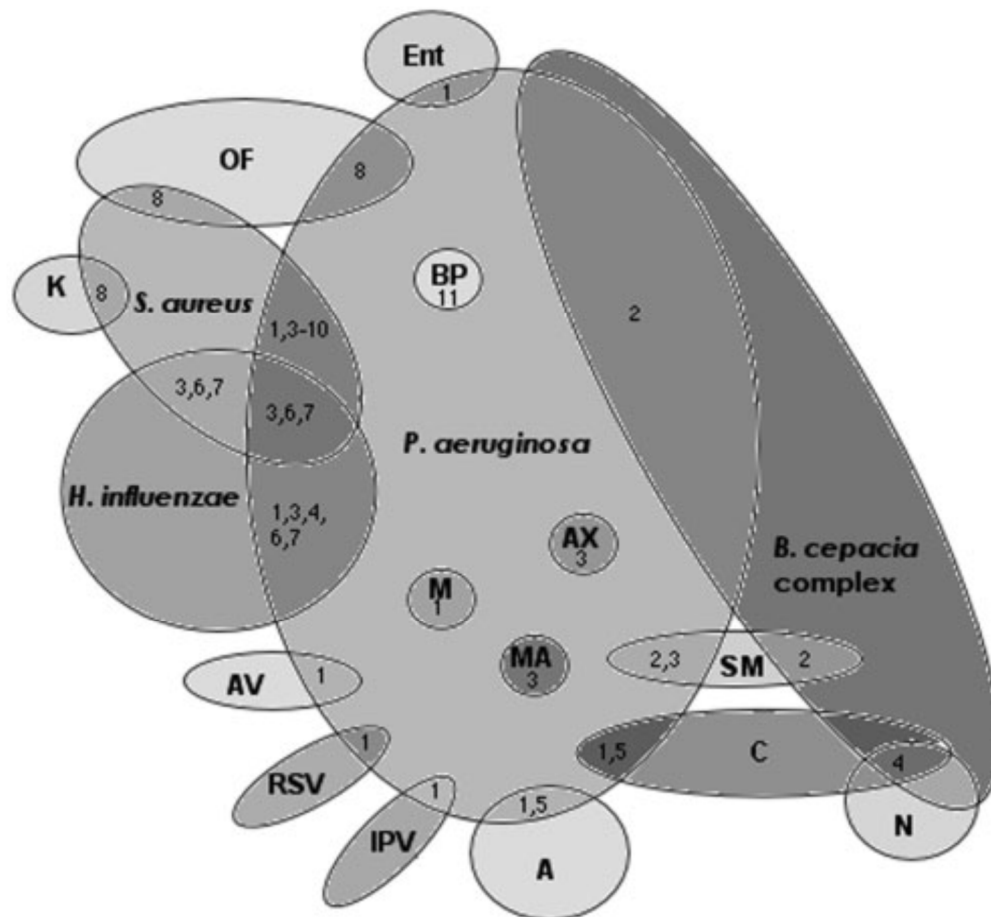


Figure 1.4. Venn Diagram of Microbial Co-Infections in the CF Lung.

Reported co-infections of microbial species within the CF lung. Co-infections do not necessarily correspond with direct interactions between the species. A, *Aspergillus* spp.; AV, adenovirus; AX, *A. xylosoxidans*; BP, bacteriophage; C, *Candida* spp.; Ent, enterobacteria; IPV, influenza and/or parainfluenza virus; K, *Klebsiella* spp.; M, mycoplasma; MA, *Mycobacterium abscessus*; N, *Neisseria* spp.; OF, oropharyngeal flora; RSV, respiratory syncytial virus; SM, *S. maltophilia*. Numbers refer to references: 1, (Petersen et al., 1981); 2, (Lambiase et al., 2006); 3, (Wahab et al., 2004); 4, (Moore et al., 2005); 5, (Burns et al., 1998); 6, (Hoiby 1974); 7, (Lording et al., 2006); 8, (Santana et al., 2003); 9, (Alvarez et al., 2004); 10, (Anzaudo et al., 2005); 11, (Ojeniyi et al., 1991). Taken from (Harrison 2007).

1.6. Clinical relevance of bacterial fungal interactions

The main reason for these studies is to discover the clinical relevance of such interactions, and to examine whether they impact upon patient health. If it is discovered that patient health and mortality is negatively impacted by mixed infections, it could inform us on the best approach to take in patient treatment.

Amin *et al.* in 2010 looked at patients with persistent *A. fumigatus* infections (defined as two or more sequential *A. fumigatus* sputum or bronchoalveolar lavage cultures within a year) via a retrospective study of 230 patients below 19 years of age (Amin *et al.*, 2010). It was found that *P. aeruginosa* was more prominent in those infected with *A. fumigatus*, with 54.1% being co-infected compared to 44.1% in an *A. fumigatus* negative group. Moreover, this combination of microbes correlated with decreased lung function.

Investigations revealed that in a combined group of the chronically and intermittently colonized patients, 86.4% of *C. albicans* - colonized patients were co-colonized with *P. aeruginosa*, whereas *P. aeruginosa* was only present in 31.1% of the control group who did not have a history of *C. albicans* colonization (Chotirmall *et al.*, 2010). Meanwhile, Azoulay *et al.* in 2006 found that in mechanically ventilated patients suffering from respiratory failure, airway infection by *C. albicans* increased the risk of subsequent infection by *P. aeruginosa* (Azoulay *et al.*, 2006).

Ultimately we still have some way to go in terms of clinical data regarding these interactions and CF, but as these studies amongst others have demonstrated, the impact of mixed interactions seems to be of substantial importance.

1.7. Physical Interactions between bacteria and fungi

Physical interaction in which the bacteria and fungi come into direct contact with one another is a way in which they can influence each other's behaviour. For the bacteria, there are multiple possible advantages from such a relationship with the fungi. (1) The fungi can be utilised as a source of nutrients (e.g. fungal cell wall, fungi-secreted products) for the bacteria to scavenge from. (2) Bacterial antagonism of fungi can be enhanced by facilitating the concentration of antifungal compounds released by bacteria in the area of fungal growth. (3) The bacteria can use the movement of the fungi within the environment to assist their own growth into new niches. (4) Assistance in bacterial-fungal synergy for the breakdown of complex substrates (Hogan. 2009). Figure 1.5, adapted from Frey-Klett *et al.* shows the various mechanisms by which physical interactions occur.

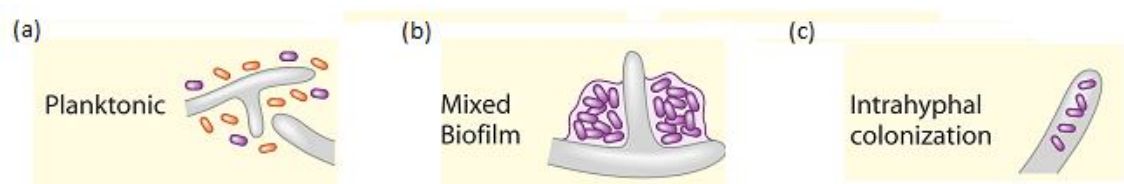


Figure 1.5. Physical interactions between bacteria and fungi.

(a) Planktonic interactions in which bacteria and fungi co-exist within an environment without a direct association. (b) Mixed biofilm in which bacteria and fungi both co-exist within the extracellular matrix, with the potential for physical interaction greatly increased. (c) Intra-hyphal colonization in which bacteria have infected and live within fungal hyphae (Frey-Klett *et al.*, 2011).

Mowat and colleagues showed experimentally that *P. aeruginosa* cells adhere to the filamentous structure of *A. fumigatus* or to the hyphae of *C. albicans* (Mowat *et al.*, 2010). For this, mature *A. fumigatus* biofilms were exposed to *P. aeruginosa* overnight. The biomass of mature biofilms following this treatment was minimally affected (15.2% of the untreated control) by the presence of *P. aeruginosa*. Scanning electron microscopy (SEM) showed type strain PA01 adhering to the biofilm structure of *A. fumigatus* biofilm as both single cells and as micro colonies (Figure 1.6). It was also demonstrated that PA01 had an inhibitory effect on the growth of *A. fumigatus* biofilm within co-culture. For example, when *A. fumigatus* conidia were grown overnight with live *P. aeruginosa* cells, the resulting fungal biomass was 14.5% ($p < 0.001$) in comparison with untreated controls. However, when mature *A. fumigatus* biofilm was treated with live *P. aeruginosa* cells, the resulting fungal biomass after overnight incubation was not significantly affected, showing 84.8% ($p > 0.05$) biomass in comparison with untreated control. This effect on *A. fumigatus* biofilm formation was generally observed over 9 different strains of *P. aeruginosa* (Mowat *et al.*, 2010).

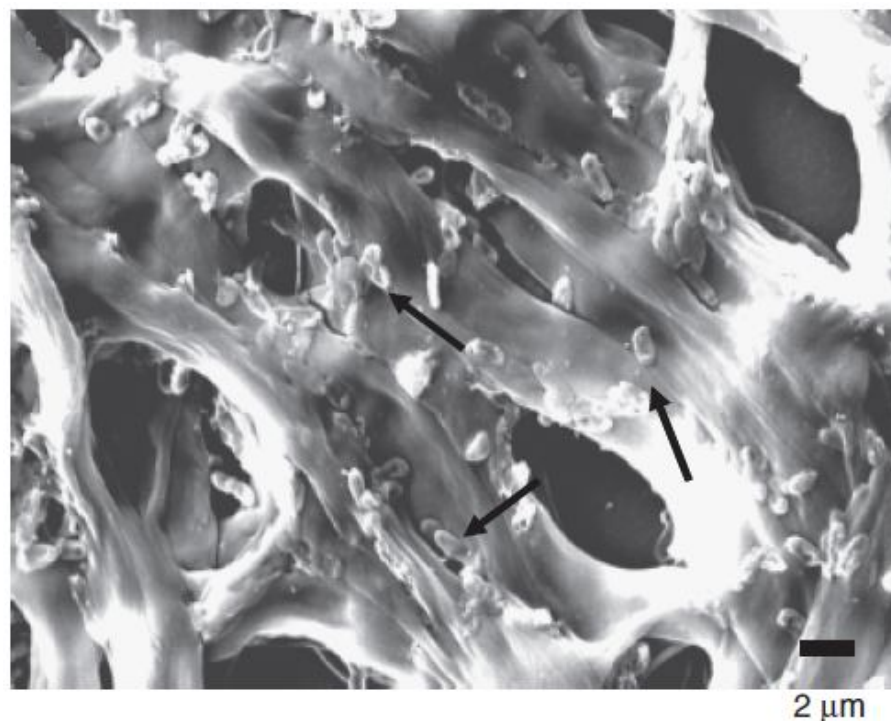


Figure 1.6. SEM evaluation of interaction between *Pseudomonas aeruginosa* PA01 and *Aspergillus fumigatus* Af293 biofilms.

Bacterial cells were shown to adhere throughout the dense intertwined filamentous structure of *A. fumigatus*. Scale bar represents 2 μm . Arrows indicate examples of *P. aeruginosa* adhering to the *Aspergillus* hyphae. (Mowat *et al.*, 2010)

In terms of competition between these organisms for resources within the host, this could be important in giving an advantage to bacteria over the fungi with bacteria exploiting the fungi for nutrients and by-products. It is also a somewhat specific process. For example, *P. aeruginosa* can adhere to *C. albicans* in the hyphae form and cause fungal killing, but it is not capable of doing this to the yeast form. Other bacterial species such as *Burkholderia cenocepacia* are also capable of this (Jarosz *et al.*, 2011).

1.8. Indirect interactions between bacteria and fungi

Within environments such as the microbiota of the CF lung, competition between various species is mediated by cell-to-cell signalling, or quorum sensing. Quorum sensing is mediated by signalling molecules (e.g. acylated homoserine lactones from Gram-negative bacteria, processed oligo-peptides for Gram-positive bacteria and farsenol for fungi) (Watnick and Kolter 2000), which regulate gene expression of genes associated with biofilm formation in response to fluctuations in cell-population density (Miller and Bassler 2001). *P. aeruginosa*, for example, uses quorum sensing to control processes such as biofilm formation, swarming motility and cell aggregation (Erickson *et al.*, 2002). Molecules associated with quorum sensing such as N-butyryl-L-homoserine lactone (C4-HSL) and N-butyryl-L-homoserine lactone (C4-HSL) have been isolated from the sputum of CF patients (Singh *et al.*, 2000). *P. aeruginosa* utilises a quorum sensing system called the *LasI/LasR-RhII/RhIR* virulence system (Figure 1.7).

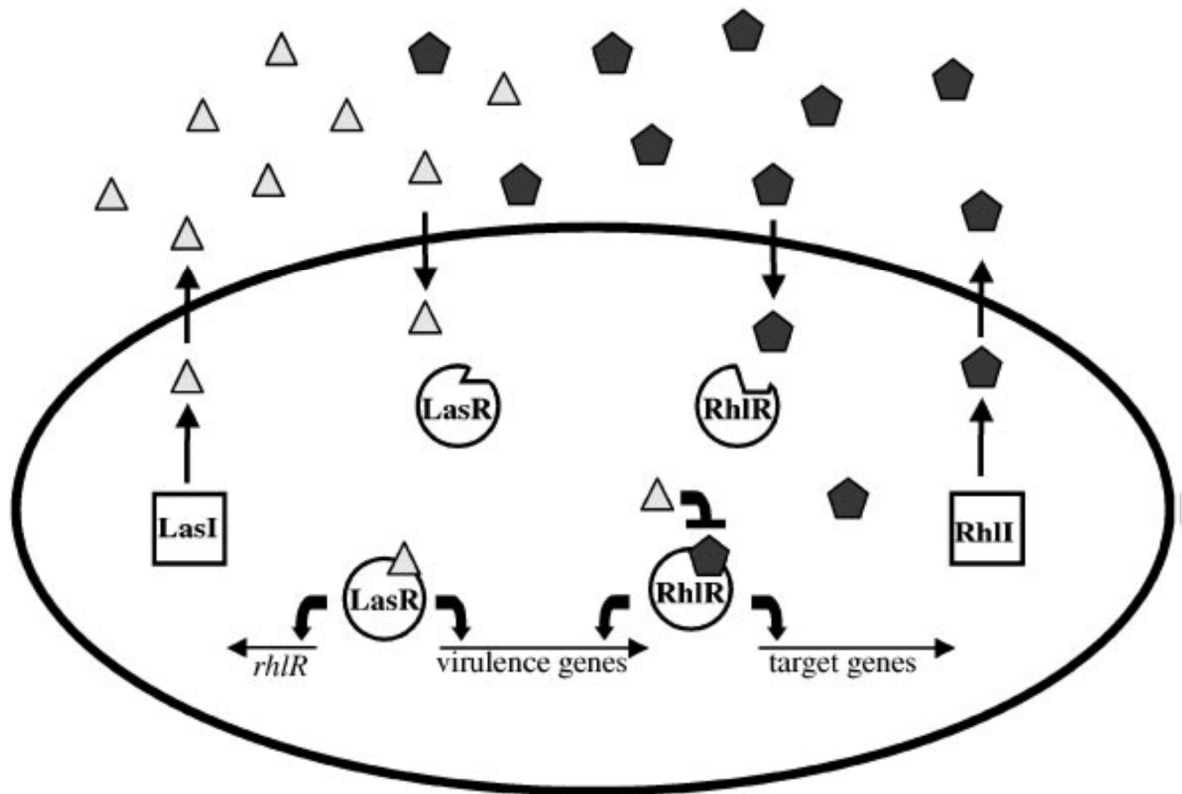


Figure 1.7. Quorum sensing in *Pseudomonas aeruginosa*.

LasI protein is responsible for producing the homoserine lactone signalling molecule *N*-(3-oxododecanoyl)-homoserine lactone (represented here by triangles). *RhII* protein synthesizes *N*-(3-butyryl)-homoserine lactone (represented here by pentagons). When the *LasI*-dependent autoinducer signal molecules rise to a critical level, they are bound by the *LasR* proteins. This triggers the binding of the *LasR*-autoinducer complex to virulence factor promoters (such as *LasB*, encoding elastase) and stimulates virulence transcription. Subsequently, the *LasR*-autoinducer complex also induces the transcription of *rhIR*, which activates a second quorum sensing circuit. *RhIR* bound to the *RhII*-directed autoinducer activates another subset of *LasR* activated virulence factors and several other target genes not encoded for by the *Las* system. The oval represents a *P. aeruginosa* cell. (Miller and Bassler 2001)

The influence of quorum sensing on bacterial-fungal interactions can be shown from work done by the Ramage group in 2010. The *LasI/R* quorum sensing system was demonstrated to have a role in the inhibition of *A. fumigatus* biofilm formation. Knockout mutants of PA01 were used for this purpose: PA01: Δ *LasI*, which was unable to produce homoserine lactones, and PA01: Δ *LasR*, which was able to synthesize homoserine lactones, but could not respond to them. Direct co-culture of these mutants with *A. fumigatus* did not inhibit biofilm growth (58.3% and 52.6% respectively) to the same extent as wild-type PA01 (22.9%) (Mowat *et al.*, 2010).

These interactions can be both beneficial and detrimental to the microbes involved in them. Bacteria or fungi can provide the other with compounds which enhance growth and survival, causing up regulation of virulence determinants, or they can negatively impact upon survival and cause virulence down regulation.

1.9. Examples of bacterial-fungal interactions and their impact on virulence

Although the overall field of bacterial fungal interactions is still in relative infancy, multiple studies have been done into various examples of such interactions and how they affect the virulence of the species involved.

One example of virulence up regulation by bacteria in the presence of fungi is that of *P. aeruginosa* up regulating phenazine toxin production when in the presence of *C. albicans* (Gibson *et al.*, 2009). It was shown in further studies that *LasR* knockout mutants (able to produce homoserine lactones, but not respond to their presence) would produce high levels of phenazine when exposed to both *C. albicans* directly or to a *C. albicans* secreted molecule called farnesol (Cugini *et al.*, 2010). Phenazine is associated with antibiotic properties against fungi such as *C. albicans* (Morales *et al.*, 2010). This virulence factor is capable of causing damage to the host lung, so therefore increased expression within the respiratory tract would contribute to morbidity in CF patients.

It has also been demonstrated that bacterial peptidoglycan-like molecules called muramyl dipeptides have been detected in human serum which could explain the increased virulence of *C. albicans* in the presence of bacteria (Xu *et al.*, 2008). These molecules are known to induce hyphae formation in *C. albicans*.

The stage at which bacteria and fungi infect the host temporally could be one factor influencing their interactions. For example, whether invasive fungi enter an environment already colonized by bacteria or vice versa. An investigation utilising an animal model (Neely *et al.*, 1986) demonstrated that *P. aeruginosa* infection preceding *C. albicans* infection within burn wounds on mice lead to an increase in mortality within the mice (Peleg *et al.*, 2010). Mice treated with a sublethal inoculum of *P. aeruginosa* before being treated with a sublethal inoculum of *C. albicans* showed 60% mortality compared to 10% in those mice treated only with *C. albicans*. It was also inferred from these experiments that the death was due to *C. albicans*. The *P. aeruginosa* virulence factor *LasB*, or PE, was implicated in causing this up regulation of virulence. Theoretically, if this was found to be the case with *A. fumigatus* as well as other bacteria, and these fungi infected the CF lung following *P. aeruginosa*, it could be hypothesised that an increased risk to the health of the CF lung could arise.

Not all interactions between fungi and bacteria involve inhibition of the others growth and physiology. However, one example of this is *P. aeruginosa* promoting the survival of *C. albicans* by secreting the extracellular signalling molecule 3-oxo-C12-homoserine lactone (3OC12HSL), which inhibits the hyphae growth of *C. albicans* (Leclair and Hogan 2010). This prevents bacterial killing of *C. albicans* as *P. aeruginosa* requires it to be in the hyphal form for killing to occur. This may also indirectly assist *C. albicans* survival in that its response involves utilising the Ras1-cAMP-controlled signalling pathway which induces oxidative stress resistance, giving *C. albicans* an increased resistance against the phagocytic cells of the immune system. Due to the high presence of *LasR* mutant strains within the CF lung (Hoffman *et al.*, 2009), this must be taken into consideration for CF bacterial fungal interactions.

In terms of further studies, the effects of fungi other than *C. albicans* on *P. aeruginosa* are of particular interest. The influence of the prominent *A. fumigatus* on *P. aeruginosa* should be high priority.

1.10. Conclusions

Despite the progression of CF treatment being a cause for celebration, there is still much work to be done, to elucidate all the factors involved in CF. The many interactions and consequences of bacterial and fungal interactions within the CF lung is one aspect that needs further investigation. The data that already exists in this area both *in vitro* and *in vivo* certainly points in this direction, as the virulence of pathogenic species that impacts upon patient mortality are affected by these interactions. This project investigates a hypothesis that co-infection with *P. aeruginosa* and *A. fumigatus* will lead to an increase in virulence in *P. aeruginosa* due to interaction with *A. fumigatus*, and utilises multiple methods to test this theory.

1.11. Aims of Study

The aims of the study were as follows:

1. To determine how *A. fumigatus* and *P. aeruginosa* interact phenotypically.
2. To evaluate the effect of the presence of *A. fumigatus* biofilm on the virulence of *P. aeruginosa* via a surrogate *Pseudomonas* virulence marker, elastase, at a biochemical and molecular level and how this in turn impacts upon a human lung epithelial cell line.
3. To determine whether *A. fumigatus* confers an increase in pathogenicity on *P. aeruginosa* within a wax worm animal model.

2 Materials and Methods

2.1. Growth and maintenance of bacterial species

Two type strains (PA01 and PA14) and two clinical isolates from the same patient (PA 06.72747A [mucoid] and PA 06.72747C [non-mucoid]) were used throughout this study (University of Calgary Foothills Hospital). PA01 Δ LasI (unable to produce homoserine lactones) and PA01 Δ LasR (unable to respond to homoserine lactones) were also used as virulence controls (kind gift from Prof Paul Williams, University of Nottingham). Finally, *Burkholderia cenocepacia* K56-2 was used as a clinical CF comparator to *P. aeruginosa*.

2.1.1.1. Growth and maintenance of bacterial strains

The relevant bacterial strains (PA01, PA14, PAATTC27853, PA 06.72747A, PA 06.72707B, PA 06.72747C and PA 06.72707A) were taken from beads (Pro-lab Diagnostics) kept in frozen stocks at -80°C and streaked out onto Luria Broth (LB [Sigma, Poole, UK]) plates before overnight incubation under static conditions at 37°C. Plates containing bacteria were stored at 4°C, and were maintained by sub-culturing the bacteria onto new LB plates on a weekly basis.

2.1.1.2. Standardisation of bacteria

Bacterial colonies were taken from LB plates using a plastic loop before inoculation into 10ml of LB media. This was incubated overnight at 37°C in an orbital shaker (IKA®, KS4000i control, Germany) set to 150rpm. Following this, the bacteria were centrifuged (MSE Centaur 1, UK) to pellet, before washing with sterile PBS. The washed cells were then resuspended in 10ml of sterile PBS before being standardized to an optical density of 0.3 at 492nm using a spectrophotometer (Fisher Scientific, Colorimeter model 45, USA), which corresponds to 1×10^8 cells/ml. A 1:100 dilution was made into media in order to produce the desired standardised bacterial concentration of 1×10^6 cells/ml for experimental work.

2.1.1.3. Growth Curves for *Pseudomonas* species

The six selected strains of *P. aeruginosa* were adjusted to 1×10^6 cells/ml in RPMI-1640 media and 200 μ l of each strain was placed into six wells of a flat-bottomed 96-well plate. The plate was placed in the plate reader (FLUOstar Omega, BMG Labtech, UK) and optical density readings were taken at 492nm every hour for 24 h, with shaking in between each reading to prevent the bacteria from adhering to the surface of the plate and creating a biofilm. The results were standardized against a blank control containing sterile RPMI-1640 media.

2.1.2. Growth and maintenance of *Aspergillus* species

2.1.2.1. Maintenance of *Aspergillus*

All work was carried out within an MDH Class I fume hood in order to contain *A. fumigatus* spores within the same environment. One hundred microlitres of Af293 conidia were pipetted onto a Saboraud Dextrose Agar (SAB) plate and spread evenly across the surface using a cell spreader, wherein they were incubated for four days at 37°C under static conditions. After four days, the surface of the plate was covered in an *A. fumigatus* lawn containing the fungal conidia. These conidia were then harvested as previously described (Rajendran *et al.*, 2011).

Briefly, phosphate buffered saline (PBS) and Tween-20 (0.25% Tween-20 to 99.75% PBS) was used to resuspend conidia from the *A. fumigatus* lawns. To do this, 5ml of PBS-Tween was pipetted onto the surface of the plate before gentle rotation to allow the PBS-Tween to move across the surface of the plate and gather conidia. The conidia were then collected in a Falcon tube and then standardised to 1×10^8 spores/mL by spore counting using a Neubauer haemocytometer. Fresh conidia were harvested approximately every two weeks, or more frequently as required.

2.1.2.2. Preparation of *Aspergillus fumigatus* biofilms

Live and dead *A. fumigatus* biofilms were required in order to determine their effect on *P. aeruginosa*. In order to prepare these biofilms, Af293 conidia were standardized to 5×10^5 conidia/ml in RPMI-1640 before addition of 500 μ l of conidia to the wells of a 24-well plate (Corning Costar, UK). The conidia were incubated under static conditions in a 37°C incubator (Labnet Vortempt 1550, UK) for 8, 12, 24 and 48 h.

To prepare killed *A. fumigatus* biofilms, excess media was removed from the biofilm before washing once with 500 μ l of PBS. The biofilm was then treated with 500 μ l of 70% ethanol and incubated at room temperature for 10 min. Following this, the ethanol was removed and the biofilm was washed with PBS (x3) in order to remove residual alcohol. The biofilm was left to dry for 10 min before addition of bacterial culture. Live *A. fumigatus* biofilms were prepared similarly, but without any ethanol treatment.

2.1.2.3. Preparation of disrupted *Aspergillus fumigatus* biofilms

Dead 24 h Af293 biofilms were prepared as detailed in 2.1.2.2. The biofilms were then removed from the surface of the plate using a cell scraper. Disrupted biofilm in RPMI-1640 media was then transferred to a 2ml Micro tube (Sarstedt, Germany), and 0.5mm glass beads (Soda Lime) (Biospec, USA) were added to the specified level before being placed in a bead beater (Mini-Beadbeater-1, Biospec, USA) for 3 x 30 s at 4800 oscillations/min. The micro tubes were centrifuged for 2 min at 3000rpm in order to separate the disrupted biofilm from the glass beads.

2.2. Physical interactions on agar plates

2.2.1. Indirect growth of *P. aeruginosa* in the presence of *A. fumigatus*

In order to show how *P. aeruginosa* and *A. fumigatus* respond to the presence of one another when growing on solid media, RPMI-1640 agar was prepared by adding 1.5% Agar No. 2 (Bioconnections, UK) to RPMI-1640 powdered media and autoclaving. *P. aeruginosa* was adjusted to 1×10^6 cfu/ml in RPMI-1640 media and a cotton swab was used to draw a straight line vertically across the plate. *A. fumigatus* was adjusted to 5×10^5 conidia/ml in RPMI-1640 media and a cotton swab was used to draw a straight line horizontally across the plate. These were left to dry for 10-15 min at room temperature before being placed in a 37°C incubator. Interactions were recorded through digital photography after 24 h in order to visually demonstrate the phenotypic interactions between the two species.

2.2.2. Filter discs and zones of inhibition

Zones of inhibition are used to quantitatively demonstrate inhibition on solid media. 10µl of *P. aeruginosa* at 1×10^6 cfu/ml in PBS, or *A. fumigatus* at 5×10^5 conidia/ml in PBS were used to impregnate sterilised filter discs before being incubated at 37°C for 24 h. Following this, three discs were transferred to each RPMI-1640 plate containing the appropriate microbial lawn. For *A. fumigatus* discs, this was 20µl of *P. aeruginosa* at 1×10^6 cells/ml spread across the surface. For *P. aeruginosa*, this was 20µl of *A. fumigatus* at 5×10^5 conidia/ml spread across the surface. Following 24 and 48 h, zones of inhibition were measured as mm of inhibition around the filter discs, with a mean being taken of the three discs per plate.

2.3. Elastin-Congo Red Assay

The Elastin-Congo Red (ECR) assay was used as a biochemical method of quantifying the phenotypic expression of the virulence factor elastase, which acted as a surrogate marker to quantify increased virulence, i.e., this allowed *P. aeruginosa* virulence expression in response to various conditions related to *A. fumigatus* to be measured and quantified.

2.3.1. Collection of supernatants for use in the ECR assay

In order to analyse the effect of *A. fumigatus* biofilms on *P. aeruginosa* and *B. cenocepacia* elastase production, the supernatants produced by these bacteria were collected. Bacteria were grown overnight in LB broth and then centrifuged at 3000rpm for 10 min in order to form a pellet. The pellet was washed using PBS, then resuspended in PBS before adjusting the optical density to 0.3 at 492nm, corresponding with 1×10^8 cells/ml. Bacteria were then adjusted to 1×10^6 in RPMI-1640 media. 500µl was added to wells of a 24-well flat bottomed plate, in which live and dead *A. fumigatus* biofilms had been prepared (see 2.1.2.2). Empty wells were used for a control without the influence of *A. fumigatus*. The bacteria were incubated under static conditions at 37°C with the *A. fumigatus* for 24 h. Live and dead *A. fumigatus* were also incubated with RPMI-1640 to control for residual endogenous elastase activity. An RPMI-1640 negative control containing no bacteria was also included. Following this, the supernatants were collected using a 2ml syringe, and were filtered using a 0.45µm filter and stored in Eppendorf tubes at -80°C until they were thawed and used for the ECR assay.

2.3.2. Elastin-Congo Red Assay Protocol

This assay was adapted from methodologies described elsewhere (Pearson *et al.*, 1997; Caballero *et al.*, 2001; Schaber *et al.*, 2004) and optimised as described in the results section. Elastin Congo-Red powder (Sigma-Aldrich, UK) was dissolved in distilled water to produce a stock solution of ECR at 100mg/ml. This was then further diluted in 0.1mM Tris-HCl buffer (pH 8.0) to produce 20mg/ml ECR buffer. In a round-bottomed 96-well plate, 100µl of ECR buffer was added to each well (giving the optimized ECR concentration of 10mg/ml) along with 100µl

of microbial supernatant. The 96-well plate was then incubated for 6 h in a 37°C incubator and shaken at 250rpm.

Following this, the samples were mixed via pipetting to resuspend ECR powder before the plates were centrifuged for 3 min at 500rpm using an ALC PK 120R refrigerated centrifuge in order to separate the insoluble ECR powder from the soluble red pigment associated with elastase activity. In cases where this had not been sufficient for full pelleting of the ECR powder, the plates were centrifuged again as necessary. 100µl of supernatant was taken from each well and transferred into a corresponding empty well in another plate in order for absorbance readings to be carried out using the plate reader (FLUOstar Omega) at 492nm.

2.3.3. Elastase Standard Curve

For each experiment, an elastase standard curve was produced in order to translate the optical density readings into quantitative data, i.e. µg/ml elastase production. This was achieved by adding 100µl of elastase (Sigma-Aldrich, UK) at a known concentration to 100µl of sterile RPMI-1640 media and then serially diluting two-fold across the plate giving a range of concentrations between 64µg/ml and 0.125µg/ml (64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125µg/ml). The assay was processed as described in 2.3.2 with ECR substrate.

2.4. Cell Culture

A human alveolar cell line (A549) was grown as an adherent monolayer in a humid 5% CO₂/ air atmosphere at 37°C.

2.4.1. A549 Cells

The A549 tumour-cell line was developed in 1972 by D.J. Giard who initiated the cell line from a human alveolar cell carcinoma of a 58-year old Caucasian male (Lieber *et al.*, 1976). These cells are human alveolar basal type-II epithelial cells (also known as great alveolar cells, or granular pneumocytes), making them of interest to CF studies in which the lung frequently becomes chronically infected with *P. aeruginosa*, *A. fumigatus* and other pathogens (Lyczak *et al.*, 2002).

2.4.2. Growth and Maintenance of the A549 Cell Line

The A549 cells were initially taken from frozen stocks kept in liquid nitrogen at -196°C. The cells were thawed rapidly in a 37°C water bath with gentle agitation. They were then added slowly to 10ml of Dulbecco's Modified Eagles Medium (DMEM) (Sigma D6429) which had been supplemented with 10% Foetal Calf Serum (FCS) containing 1g/L glucose and 2mM L-glutamine (DMEM/10%FCS) before transfer to a T75 Flask (Cell Star) in which the cells were cultured. This was maintained in 95% humidity at 37°C and 5% CO₂ until they reached a density of 2x10⁶ cells/flask.

At this stage the cells were removed from the surface of the flask by using the serine protease trypsin (Invitrogen, Paisley UK). Firstly, the medium was removed from the adherent cells within the flask and then 10ml of Dulbecco's PBS (pH 7.3-7.4) was used to wash the cell monolayer twice. Following washing, 5ml of Trypsin/EDTA (Gibco) was added to the flask. The flask was then moved to the 37°C incubator and monitored frequently over 5-15 min until the cells had detached from the flask surface. At this point, 15ml of RPMI-1640/10% FCS was added to inhibit the enzymatic action of trypsin before it could cause damage to the cell line. The cells were then transferred to a 25ml universal tube and

centrifuged at 80g for 5 min in order to pellet the cells and separate them from most of the Trypsin/DMEM. The supernatant was removed leaving less than 0.5ml covering the pellet, and this was resuspended following the addition of 1ml further of RPMI-1640/10%FCS.

2.4.3. Trypan Blue

Trypan blue is a dye that is used to distinguish whether cells are alive or dead upon staining. Treated A549 cells were dyed using trypan blue and the number of dead cells within the field of cells under a microscope was counted. This allowed the viability of the cells after treatment to be further verified, as an additional measure outside of the Alamar Blue assay.

2.4.4. Cell Microscopy

The cell count was then determined using a haemocytometer, viewed under light microscope. In brief, 20 μ l of the cell culture was gently mixed with 10 μ l of trypan blue dye, with 10 μ l of this being placed under a cover slip on the haemocytometer. The cells within the centre square were counted, with cells stained blue by the Trypan blue being disregarded. The cells were then counted using a haemocytometer and standardised to a concentration of between 2×10^5 and 5×10^5 cells/ml for seeding a T75 flask. The cells were then monitored daily using inverted phase contrast microscopy.

2.4.5. A549 Cell Stock Preparation

In order to preserve stocks for future work and in case the flask of cell culture being maintained was lost due to high passage number, accidents or contamination, some cells were frozen down again. These A549 cells were grown until they reached 80% confluence. At which point they were removed from the flasks by trypsinisation, as described above. After inhibition of trypsin and centrifugation described above the cells were adjusted to a concentration of between 1×10^6 and 2×10^6 cells/ml in DMEM/10%FCS, and then mixed gradually with an equal volume of 2 parts dimethylsulfoxide (DMSO) to 7 parts DMEM to 1 part FCS.

2.4.6. Alamar Blue Assay

The Alamar Blue cell viability assay (Invitrogen, Paisley UK) is used to quantify cytotoxicity of various agents upon cell lines (O'Brien *et al.*, 2000). This assay utilises Alamar Blue reagent, a water-soluble dye which undergoes a colour change based on the metabolic activity of the cells being tested. A REDOX (reduction-oxidation) reaction caused by growing cells reduces Alamar Blue dye causing a colour change from blue to pink which is measurable using a plate reader, with high reduction corresponding to low cytotoxicity and high oxidation corresponding to high cytotoxicity.

2.4.6.1. Alamar Blue Protocol

A549 cells were seeded into the wells of 24-well flat bottomed cell culture plates (Costar, UK) at 1ml of 1×10^5 cells/ml in RPMI-1640 and left overnight at 37°C 5% CO₂. The following day, microscopy was used to determine that the cells had successfully adhered to and grown in the wells, before the media was removed and replaced with 500µl of fresh media, or 500µl of media combined with an agent being tested for cytotoxicity (e.g. bacterial and fungal supernatants). Each experimental condition was tested in triplicate wells on three separate occasions. The plates were then returned to the incubator and left for approximately 24 h. Following this, 50µl of media was removed from each well and replaced with 50µl of Alamar Blue reagent (giving a 1:10 ratio) and left to incubate for approximately 3 h. Sterile media containing Alamar Blue reagent was used as a control. 100µl was removed from each well in triplicate, giving 9 replicates for each condition per experiment, and transferred to a 96-well flat bottomed plate (Costar). Absorbance readings were taken at both 570nm and 600nm using the plate reader (FLUOstar Omega, BMG Labtech, UK).

2.4.6.2. Alamar Blue Percentage Reduction Analysis

In order to calculate the percentage of reduction of Alamar Blue within the assay, the values recorded were inputted into an absorbance equation. This equation is stated as:

$$\text{Percentage reduction of alamarBlue} = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

In which: E = molar extinction coefficient

O1 = E of oxidized Alamar Blue at 570nm

O2 = E of oxidized Alamar blue at 600nm

R1 = E of reduced alamar Blue at 570nm

R2 = E of reduced alamar Blue at 600nm

A1 = absorbance of test wells at 570nm

A2 = absorbance of test wells at 600nm

N1 = absorbance of negative control well at 570nm

N2 = absorbance of negative control well at 600nm

By utilising these calculations, the percentage reductions of Alamar Blue for each well were determined, allowing cytotoxicity to be measured. The RPMI-1640 control well is taken as 100% percentage reduction of Alamar Blue for the purposes of these experiments, with the test wells being standardized against this.

2.4.6.3. Photography of A549 Cells

Light microscopy was carried out to visualize the effect of relevant treatments on A549 cells. This was carried out at x25 magnification on a Leitz Labovort light microscope (Leitz, Wetzlar, Germany) and photographs were captured using a Canon IXUS 220HS digital camera (Canon, Japan).

2.5. Real Time PCR Analysis

2.5.1. RNA Extraction, Quantification and cDNA Synthesis

RNA was extracted from PA01 cells grown in both the presence and absence of dead Af293 biofilm at 1×10^8 cells/ml through the use of the RNeasy mini kit (Qiagen, UK) and RNAprotect Bacteria Reagent (Qiagen), according to manufacturer's instructions. The bacterial RNA was stabilised by adding 1 volume to bacterial culture to 2 volumes of RNAprotect Bacteria Reagent. The cells were lysed using lysozyme at 1mg/ml in TE buffer before collection of RNA and subsequent purification using the RNeasy mini kit. The RNA obtained was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). 1.5 μ L of RNA from each sample was loaded onto NanoDrop pedestal. The resultant RNA was of high quality, approximating 2.0 (260/280). cDNA synthesis was achieved using high capacity RNA-to-cDNA synthesis kit (Invitrogen, Paisley UK).

Reverse transcription (RT) reaction mix was prepared as detailed in Table 2.1A. 20 μ L of RT reaction mix was aliquoted into reaction tubes which were sealed and briefly centrifuged to spin down contents and eliminate air bubbles. The tubes were kept on ice before being loaded into the thermal cycler. The thermal cycler was programmed as detailed in Table 2.1B before the reverse transcription run was carried out. cDNA was stored at -20°C before use.

Table 2.1. cDNA synthesis. A) Components of RT reaction mix (per 20 μ l reaction). B) Reverse transcription cycle conditions.

A)

Component	Volume/Reaction (μ l)	
	+RT	-RT
2X RT Buffer	10.0	10.0
20X RT Enzyme Mix	1.0	-
Nuclease-free H ₂ O	Q.S. to 20 μ l	Q.S. to 20 μ l
Sample	Up to 9 μ l	Up to 9 μ l
Total per Reaction	20.0	20.0

B)

	Step 1	Step 2	Step 3
Temperature ($^{\circ}$ C)	37	95	4
Time	60 min	5 min	∞

2.5.2. DNA digestion and RNA cleanup

In order to prevent the presence of DNA contamination within RNA samples, a DNase digestion was carried out on each sample according to the manufacturer's instructions (Qiagen). DNase I (Qiagen) dissolved in RNase-free water was used for this. ≤ 87.5 μ l RNA, 10 μ l RDD and 2.5 μ l DNase 1 stock solution were mixed and made up to 100 μ l with RNase-free water and incubated on the desktop for 10 min at room temperature before RNA cleanup was carried out following the manufacturer's instructions, resulting in RNA of higher integrity. This was done by adjusting the sample to 100 μ l with RNase-free water before adding 350 μ l Buffer RLT and mixing. 250 μ l ethanol (96%) was added to the diluted RNA and mixed well. The sample was transferred to a RNeasy Mini spin column placed in a 2 ml collection tube. This was then centrifuged for 15 s at 10,000 rpm. The flow through was discarded. 500 μ l Buffer RPE was added to the RNeasy spin

column. This was then centrifuged for 15 s at 10,000 rpm to wash the spin column membrane. This was then repeated with a further 500 µl Buffer RPE for 2 min. The RNeasy spin column was then placed in a new 1.5 ml collection tube. 30 µl RNase-free water was added directly to the spin column before centrifugation at 10,000 rpm for 1 minute to elute the RNA. The RNA was then collected and stored at -20°C.

2.5.3. qPCR

Expression levels of *LasB* were measured in relation to the housekeeping gene *rpoD*. *rpoD* is a critical housekeeping gene which encodes a sigma factor, and has previously been shown to be a good internal control for qPCR experiments in *P. aeruginosa* (Savli *et al.*, 2003). The primers were taken from Lee and co-workers (Lee *et al.*, 2011) and the thermal profile described by Sang Sun Yoon via personal correspondence (Tables 2.2). Each well contained 12.5 µL of SYBR green master mix (Invitrogen, UK), 0.5 µL of forward and 0.5 µL of reverse primers, 1 µL of cDNA and 10.5 µL of DNase/RNase free water (Qiagen, UK) to make up a total volume of 25 µL. A final concentration of 200 nM per primer was utilised.

The mean efficiencies were determined in the sample triplicates and used to adjust the cycle threshold (Ct) values. Ct values were used for the quantitative comparison of the amplification rates. After baseline subtraction, the mean Ct values of the triplicates were determined and transformed into relative quantities utilising an existing calculation formula set up in Excel (Livak and Schmittgen 2001).

Table 2.2. Primers used in this study.

A) Primers were chosen in line with previous literature. B) Primer conditions; including temperature and number of cycles.

A)

Target	Primer	Reference
LasB Forward	TTAGCACTCCTTGGCAAAAC	(Lee <i>et al.</i> , 2011)
LasB Reverse	CAGAGACAGCAGAGCACACAA	
rpoD Forward	CAAGGCTGAGAACGGGAAG	(McKimmie <i>et al.</i> , 2008)
rpoD Reverse	GGTGGTGAAGACGCCAGT	

B)

Function	Temperature (°C)	Time	Cycles	Reference
Denaturation	55	2 min	1	Personal correspondence with Sang Sun Yoon, Yonsei University College of Medicine
Denaturation	95	10 min	40	
Annealing	60	30 s		
Extension	59	1 min		
Extension	72	1 min	1	

2.6. Wax Worm Model of Infection

The wax worm model of infection uses the larval stage of the Greater Wax Moth, *Galleria mellonella*. This species of moth, from the Pyralidae family, is found across most of the world, believed to have originated in Europe, and larvae often feed on the nests of bees, making them an occasional pest species. Fully grown larvae are about 28mm in length, and are of a greyish-white colour (Figure 2.1). Upon death, rapid melanisation occurs resulting in the wax worm appearing black (Figure 2.1).

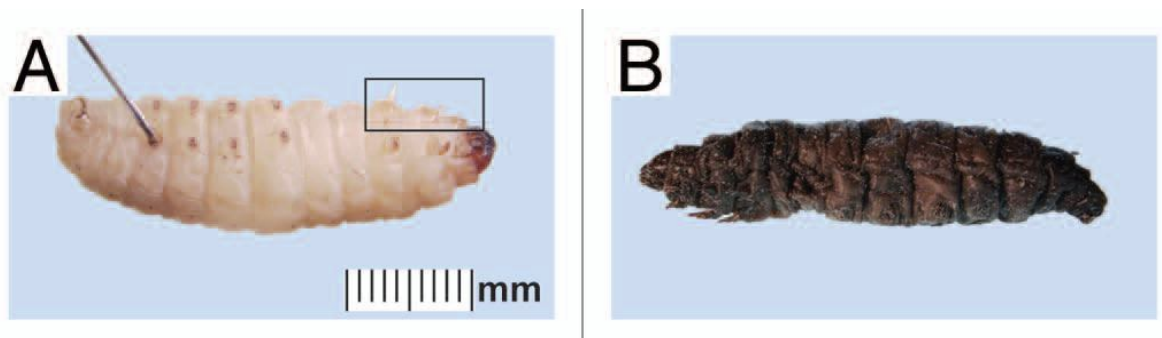


Figure 2.1. Wax worm model of infection.

Galleria mellonella is a commonly used insect model of infection. (A) Worms are typically a greyish-white colour and 28mm in size. Injections are carried out in the left hindmost pro-leg. (B) Pigmentation tends to occur when the worms become infected resulting in a characteristic colour change towards brown and eventually black.

2.6.1. Preparation of killed Af293 germlings for wax worm injection

In order to prepare killed germlings, 2×10^6 conidia/ml and lower were required. Therefore, a high number of conidia ($>1 \times 10^8$ conidia/ml) were incubated in RPMI-1640 for 8 h before centrifugation at 3000rpm. Excess media was removed and the *A. fumigatus* was resuspended in 70% ethanol before centrifugation. The pellet was then washed three times in PBS, before the conidia were counted using the haemocytometer. Visual observation was used to confirm conidia had germinated and produced germlings. The germlings were then prepared to required concentrations in PBS

2.6.2. Injection of Wax Worms with *Pseudomonas aeruginosa* and Monitoring of Survival Rates

PA14 was inoculated in LB broth for 24 h, before centrifuging to produce a bacterial pellet. This was resuspended in PBS and the optical density was adjusted to 0.3, giving 1×10^8 cells/ml. This was then serially diluted ten-fold in PBS, producing concentrations of 1×10^7 to 1×10^3 cells/ml. This corresponds with 10,000 to 1 bacteria per injection per worm, respectively. These concentrations were injected into the wax worms in measures of 10 μ l. Two negative controls were set up, including a vehicle PBS control and a mock-injected control. Five worms were prepared for each experimental condition.

During experiments, worms were kept in the fridge until very shortly before injection in order to keep them moderately inactive. Once removed from the fridge, they were kept in a Petri dish surrounded by ice in order to keep conditions cool, preventing the worms from becoming too active leading to increased difficulty in the injecting process. The syringe was sterilized using ethanol and then washed with PBS before use. A swab was used to wash the area of injection for the worms using 95% ethanol in order to prevent bacteria or fungi present on the wax worms body from entering the wound site. Injections were carried out using a 50 μ l Hamilton syringe with a 26G BD Microlance. Each worm was injected in the left hindmost proleg with care being taken to avoid causing injury or bleeding to the worm. Live and dead worms are distinguished by movement of the worms, although over a longer timescale worm's shift from greyish-white to black in pigmentation.

2.6.3. Injection of Wax Worms with *Pseudomonas aeruginosa* and *Aspergillus fumigatus* and Monitoring of Survival Rates

PA14 was prepared as detailed in Section 2.6.1 and standardized to 2×10^3 cells/ml in PBS. 8 h Af293 germlings were prepared as detailed in Section 2.6.1 and standardised to 2×10^6 germlings/ml. Bacteria and killed germlings were mixed in a 50:50 ratio and vortexed, giving 1×10^3 cells/ml PA14 and 1×10^6 killed 8 h germlings/ml. PA14 alone and 8 h Af293 germlings alone were mixed in a 50:50 ratio with PBS to give 1×10^3 cells/ml PA14 and 1×10^6 killed 8 h germlings/ml. Wax worms were injected with combinational culture and PA14, dead 8 h Af293 germling and PBS controls as described in 2.6.2 and monitored for survival.

2.7. Statistics

Statistical analysis was carried out in GraphPad Prism 4 or Excel. One-Way ANOVA and the Bonferroni post-test was utilised throughout the project. Percentage and fold change data were subjected to a natural log transformation to facilitate the use of a non-parametric test. The analysis was performed using one-way ANOVA. To determine where the statistical differences were between the groups Dunnett post-test or Bonferroni post-test were used where appropriate. On some occasions where ANOVA was not appropriate t tests were applied with Bonferroni corrections. The appropriate t test and post test were selected after analysis of the variance by f test. Survival curve analysis for wax worm survival was carried out using the inbuilt survival data analysis facility within GraphPad Prism 4.

3. Results

3.1. Media optimisation

3.1.1. Evaluation of various media for microbial growth

In order to maintain consistency in the growth medium in which *A. fumigatus* and *P. aeruginosa* were grown, several media that supported growth of both species was tested. Based on a previous report, three different types of agar were investigated: Luria broth (LB) agar, Roswell Park Memorial Institute-1640 medium (RPMI-1640) agar and Sabouraud Dextrose agar (SAB) (Mowat *et al.*, 2010). 50µl of *A. fumigatus* Af293 was pipetted onto the surface of the plates and observed over 4 days to qualitatively monitor the levels of growth. Single colonies of *P. aeruginosa* type strains PA01 and PA14 were plated onto the surface of all three agar types and how well they grew was determined based on the levels of subjective visual growth: light (+), medium (++) or heavy (+++). This subjective analysis of growth allowed for the appropriateness of media to be indicated. RPMI-1640 was shown to support moderate growth of both organisms (Table 3.1), whereas both LB and SAB variably supported the growth of *A. fumigatus* and *P. aeruginosa*. Therefore, RPMI-1640 was selected for future investigations.

Table 3.1. Qualitative level of growth observed from *Aspergillus fumigatus* and *Pseudomonas aeruginosa* on LB, SAB and RPMI-1640 agar plates.

Level of growth on agar plate		
	<i>P. aeruginosa</i>	<i>A. fumigatus</i>
LB	+++	+
SAB	+	+++
RPMI-1640	++	++

3.1.2. Planktonic growth of different strains of *Pseudomonas aeruginosa*

In order to select suitable strains of *P. aeruginosa*, seven strains were tested and planktonic growth rates assessed. This was to ensure variation in elastase production was not simply due to any given strain growing at a significantly higher rate than the others. Rates of exponential growth (Best Fit values) are as follows: 0.01657 O.D/h (PA01), 0.02038 O.D/h (PA14), 0.02409 O.D/h (PA ATTC 27853), 0.03692 O.D/h (PA 06.72747A), 1×10^{-7} O.D/h (PA 06.72707B), 0.03229 O.D/h (PA 06.72747C) and 0.01998 O.D/h (PA 06.72707A). It was shown that all strains reached exponential and lag phase at a similar rate (ANOVA and the Bonferroni post-test $p > 0.05$), with the exception of 06.72707B, which showed statistically lower growth (ANOVA and the Bonferroni post-test $p < 0.001$) than the other six strains (Figure 3.1).

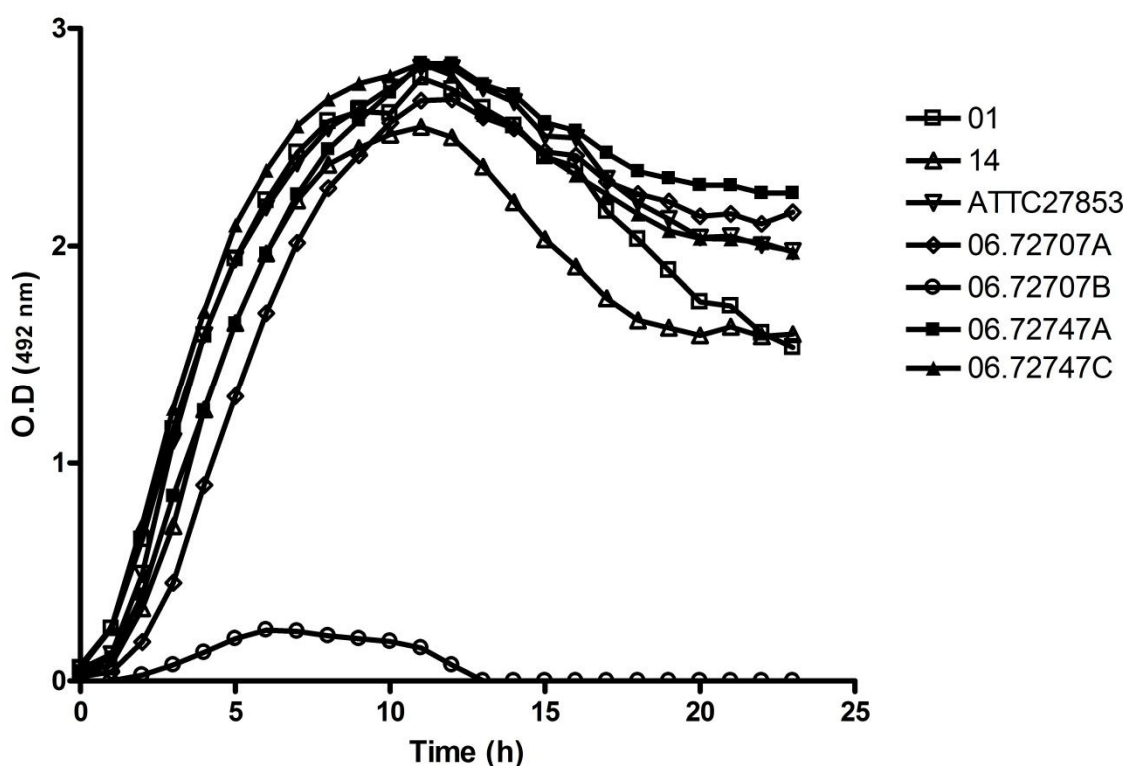


Figure 3.1. Planktonic growth rates of *Pseudomonas aeruginosa* in RPMI-1640 media.

P. aeruginosa type strains 01, 14 and ATTC 27853 and clinical isolates 06.72707A, 06.72707B, 06.72747A and 06.72747C were grown in planktonic form in RPMI-1640 for 24 h in a round bottomed 96 well microtitre plate from an initial concentration of 1×10^6 cells/ml at 37°C. Strains demonstrated similar rates of growth ($p > 0.05$) with the exception of 06.72707B ($p < 0.001$). This experiment was performed once in triplicate, with points representing the mean. $n=1$.

3.2. Phenotypic interactions of *Pseudomonas aeruginosa* and *Aspergillus fumigatus*

In order to derive insight into the interactions between *P. aeruginosa* and *A. fumigatus* on a phenotypic level, standard plating assays were utilised in order to visually describe some basic interactions between the two organisms.

3.2.1. Phenotypic interactions between *Pseudomonas aeruginosa* and *Aspergillus fumigatus*

A simple plate assay was used to show *P. aeruginosa*/*A. fumigatus* interactions together on RPMI-1640 agar. *P. aeruginosa* (PA01 and PA14) and *A. fumigatus* (Af293) were streaked onto the same plate at the same time, with the growth being monitored at 24 h and images taken (Figure 3.2). It was qualitatively observed that a small area of inhibition occurred where the line of *P. aeruginosa* was in close contact with, but not touching, the line of Af293.

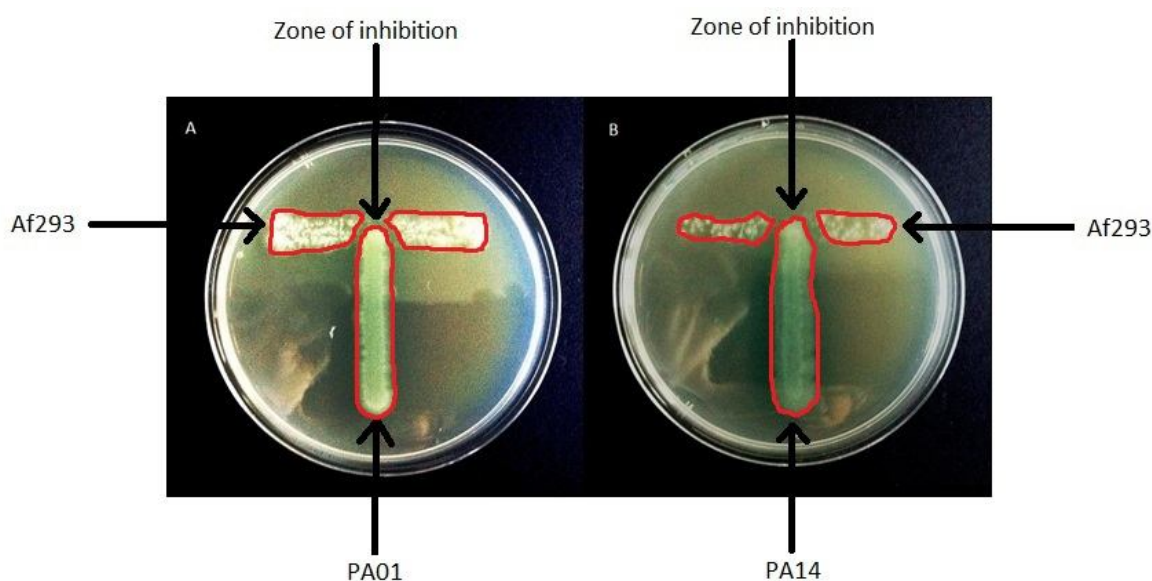


Figure 3.2. Phenotypic interactions between *Pseudomonas aeruginosa* and *Aspergillus fumigatus* on the surface RPMI-1640 agar plates.

(A) PA01 and (B) PA14 were standardised to 1×10^6 cells/ml in RPMI-1640 media and streaked once vertically onto the surface of an RPMI-1640 agar plate using a cotton swab. Af293 was standardised to 5×10^5 conidia/ml in RPMI-1640 media and a single horizontal line streaked perpendicularly but not touching, the other line. Plates were incubated at 37°C for 24 h prior to imaging. n=1.

3.2.2. *Pseudomonas aeruginosa* inhibition of *Aspergillus fumigatus*

In order to further investigate inhibitory interactions between the two organisms in a more quantitative manner, filter paper discs impregnated with *A. fumigatus* (Af293) for 24 h, were incubated on an inoculated *P. aeruginosa* (PA01 and PA14) lawn on an RPMI-1640 agar. After 24 h incubation, the resulting zones of inhibition were measured and images taken (Figure 3.3). For PA01, the zones of inhibition measured around the Af293-impregnated filter discs were 12, 16 and 10mm, giving an average of 12.67mm. For PA14, the zones of inhibition measured around the Af293-impregnated filter discs were 17, 15 and 12mm, giving an average of 14.67mm. These results indicated that the presence of *A. fumigatus* on RPMI-1640 agar caused inhibition of *P. aeruginosa* growth to a small extent.

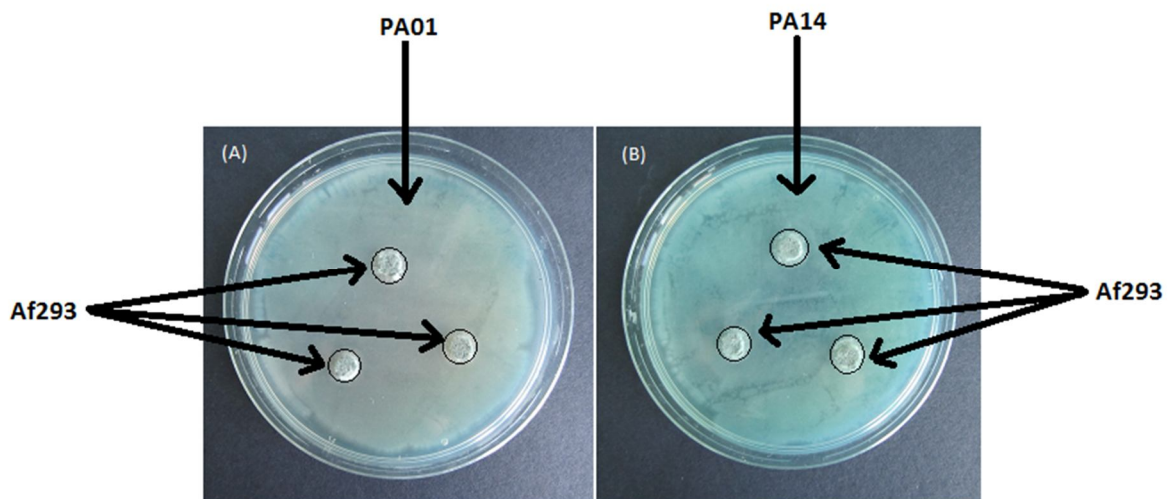


Figure 3.3. Live *Aspergillus fumigatus* filter disc growth on *Pseudomonas aeruginosa* lawns.

Filter paper discs containing *A. fumigatus* biofilms grown for 24 h at 37°C 10µl of 5×10^5 Af293 conidia/ml in RPMI-1640, were placed onto a lawn of 20µl of (A) PA01 at 1×10^6 cells/ml in PBS or (B) PA14 at 1×10^6 cells/ml in PBS. After 24 h incubation, zones of inhibition (including the disc itself) were measured in millimetres. The zones of inhibition are circled in black. n=1.

The same experimental setup was also carried out with filter discs impregnated with 24 h *P. aeruginosa* (PA01 and PA14) on a newly inoculated Af293 lawn in order to test the ability of *P. aeruginosa* to inhibit *A. fumigatus* growth. After 24h, the resulting zones of inhibition were measured and images taken (Figure 3.4). For PA01, the zones of Af293 inhibition measured surrounding the filter discs were 29, 16 and 20mm, giving an average of 21.67mm. For PA14, the zones of Af293 inhibition measured surrounding the filter discs were 18, 24 and 26mm, giving an average of 22.67mm. These results indicated the presence of *P. aeruginosa* on RPMI-1640 agar caused inhibition of *A. fumigatus* growth.

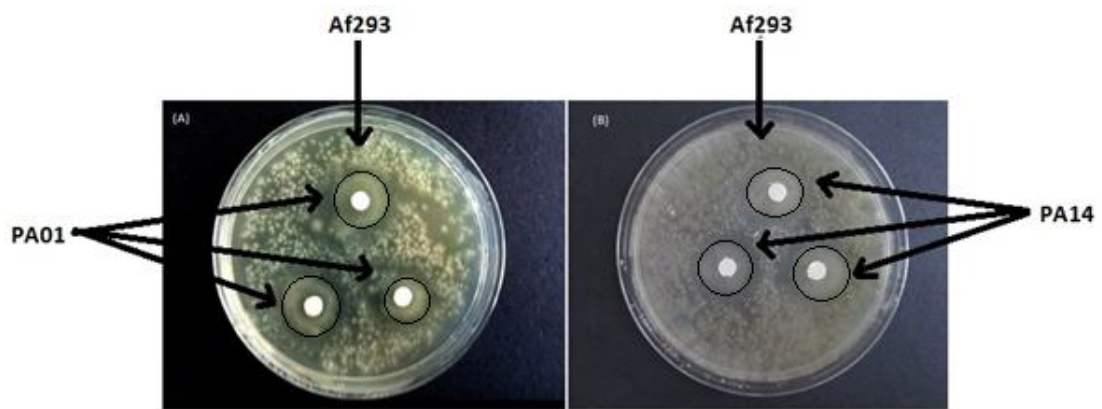


Figure 3.4. Live *Pseudomonas aeruginosa* filter disc growth on *Aspergillus fumigatus* lawns.

Filter paper discs containing (A) PA01 or (B) PA14 1×10^6 cells/ml in PBS were grown for 24 h at 37°C and placed onto a lawn of $20\mu\text{l}$ of 5×10^5 Af293 conidia/ml in RPMI-1640. After 24 h incubation, zones of inhibition (including the disc itself) were measured in millimetres. $n=1$.

3.3. Quantification of the *Pseudomonas aeruginosa* virulence factor elastase in the presence and absence of *Aspergillus fumigatus*

3.3.1. Introduction

One possible consequence of *P. aeruginosa* interacting with *A. fumigatus* is the up-regulation of virulence factors leading to increased pathogenicity within the host (Neely *et al.*, 1986; Xu *et al.*, 2008). Elastase is a key virulence factor secreted by *Pseudomonas* species, therefore, this was chosen as a surrogate marker for virulence up-regulation of the bacteria in response to fungi (Kuang *et al.*, 2011). Elastase was selected due to its clinical relevance (Hoge R. 2010), and because a biochemical assay, the Elastin Congo Red (ECR) assay, was readily available for quantifying its production (Caballero *et al.*, 2001; Schaber *et al.*, 2004).

Initial studies involved the standardisation of the ECR assay for its use in future experiments throughout this project. In addition, the ECR assay was used to demonstrate how interactions between *P. aeruginosa* and various *A. fumigatus* biofilm phases affected elastase production by *P. aeruginosa*.

3.3.2. Standardisation and validation of the ECR assay

These studies were designed to determine the optimum methodology for the ECR assay, which underpinned experiments carried out within this project. Studies were then carried out to prove that the ECR assay was suitable for measuring elastase produced by *P. aeruginosa*.

3.3.2.1. Standardisation of the ECR assay with regards to ECR buffer concentration and incubation time.

Several potential methodologies for the ECR assay with regards to concentration of buffer and incubation time have been described (Pearson *et al.*, 1997; Caballero *et al.*, 2001; Schaber *et al.*, 2004). In order to determine the optimum conditions for the purpose of these studies, a standardisation experiment was carried out (Figure 3.5). For each of the concentrations of ECR buffer tested (2.5, 5, 10, 20 and 40 mg/ml), an increase in absorbance (OD as

measured at 492nm) was recorded over time. There was an exception with 2.5 and 5 mg/ml ECR buffer, where a small decrease was observed between 6 and 7 h time points. No significant difference in absorbance occurred between the different concentrations tested with the exception of the difference between 2.5 mg/ml and 40 mg/ml (ANOVA and the Bonferroni post-test $p < 0.05$). 40 mg/ml ECR buffer consistently produced higher absorbance readings than any other concentration used, peaking at an OD₄₉₂ of 1.06 after 7 h in comparison with a OD₄₉₂ reading of 0.45 for 20 mg/ml ECR buffer after 7 h. As a suitably high absorbance reading of 0.43 was found after 6h when using 20 mg/ml ECR buffer, this concentration and period of incubation was utilised for future experiments.

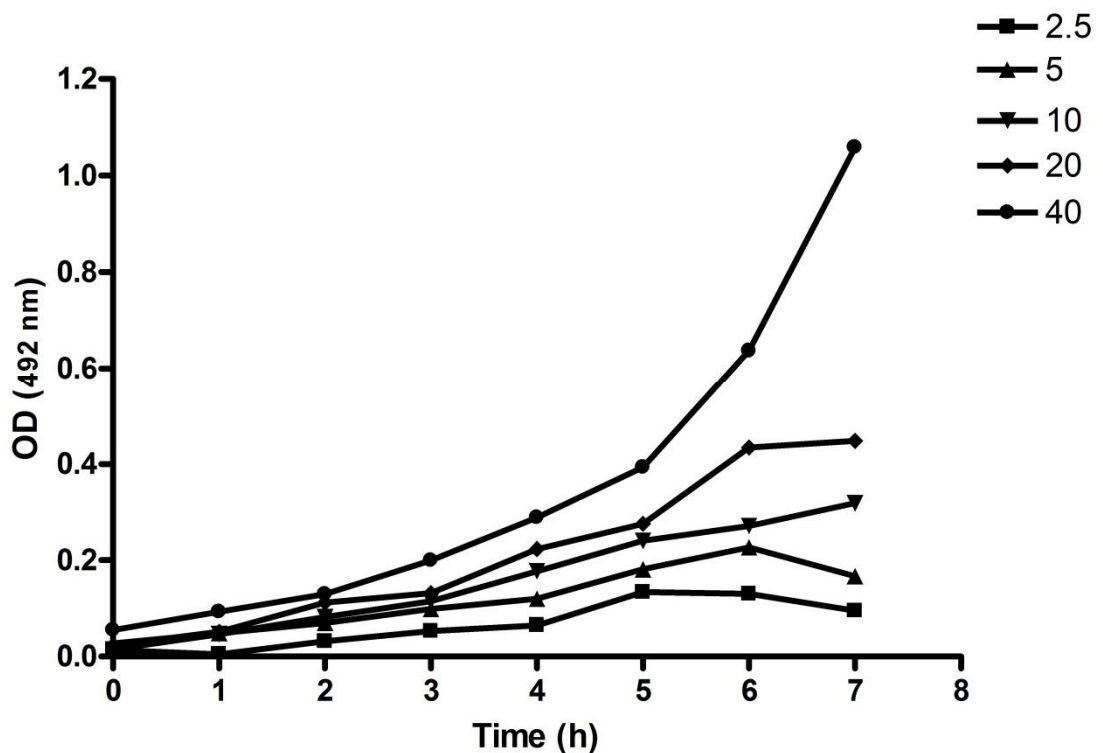


Figure 3.5. Standardisation of the ECR assay with regards to buffer concentration and incubation time.

PA14 was grown at 1×10^6 cells/ml in a final volume of 500 μ l in RPMI-1640 media in a 24-well plate for 1, 2, 3, 4, 5, 6 and 7 h before quantification of elastase via the ECR assay utilising ECR buffer concentrations of 2.5, 5, 10, 20 and 40 mg/ml Elastin-Congo Red. No significant difference ($p > 0.05$) in absorbance was observed between 24 h PA14 supernatants, with the exception of 2.5 mg/ml and 40 mg/ml ECR buffer. Each experiment was performed in triplicate, with points representing the mean. $n=1$.

3.3.2.2. Elastase Standard Curve for the ECR Assay

Quantification of the elastase produced by *P. aeruginosa* was assessed using a standard curve of known concentrations of elastase in relation to the level of soluble pigment produced. In the representative standard curve shown (Figure 3.6), a clear correlation ($R^2=0.9974$) was found between concentration of elastase and absorbance at 492nm of soluble product. The gradient of the straight line resulting from this standard curve was then used to calculate the concentration of elastase. An elastase standard curve was set up each time the assay was carried out for these studies, with the assay being deemed successful when the R^2 value of the standard curve was more than or equal to 0.95. If the standard curve varied from this, this was deemed as an internal control showing that the assay had failed.

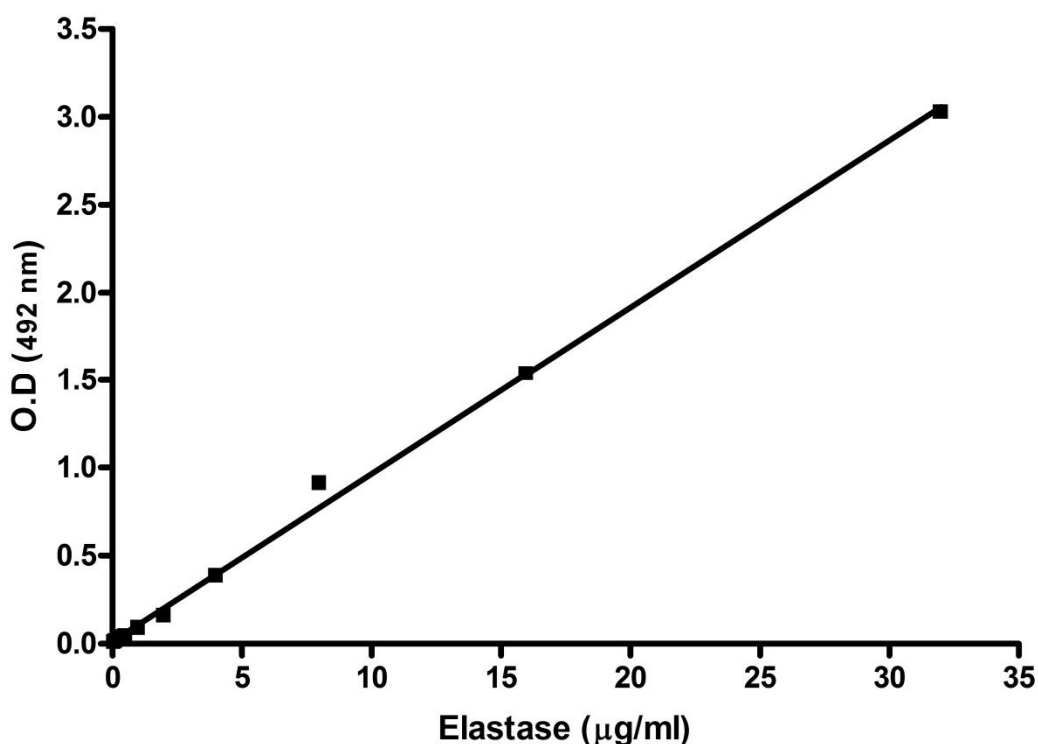


Figure 3.6. Representative ECR standard curve.

Presence of soluble ECR pigment increases as the concentration of elastase increases. Porcine pancreatic elastase was incubated in 100μl of RPMI-1640 media at various concentrations (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 μg/ml) with 100μl of 20 mg/ml ECR buffer for 6 h, before the insoluble product was removed and the absorbance of the soluble pigment read at 492nm. This data was taken from a single representative determination run in duplicate with points representing the mean. $n=1$.

3.3.2.3. Elastase production of selected *Pseudomonas aeruginosa* strains

The elastase production of several strains of *P. aeruginosa* (Chapter 2.1) was measured in order to determine whether the ECR assay could successfully detect elastolytic activity in *P. aeruginosa*, and also which strains were best suited for use within future studies.

Elastase production was shown to vary between the strains tested (Figure 3.7). PA01 showed the highest level of elastase production, with 2.51µg/ml elastase being measured in comparison to elastase production as seen in PA14 with 2.18 µg/ml, a 115.1% increase. Both type strains of *P. aeruginosa* were shown to produce greater levels of elastase than mucoid and non-mucoid clinical isolates PA 06.72747A (M) and PA 06.72747C (NM), which produced 2.03 µg/ml and 1.79 µg/ml, respectively. Mucoid and non-mucoid strains PA 06.72747A (M) and 06.72747C (NM), produced 2.03 µg/ml and 1.79 µg/ml, respectively. Type strain PA ATTC 27853 was discounted from further studies as it produced negligible amounts of elastase (0.01 µg/ml), as was mucoid and non-mucoid clinical isolates PA 06.72707A (NM) and PA 06.72707B (M) as the mucoid strain failed to produce elastase, with a mere 0.01 µg/ml elastase being measured. As mucoid and non-mucoid clinical isolates were being used for comparison studies within the same strain, PA 06.72707B (M) being discounted from future studies meant that PA 06.72707A (NM) was also discounted. This was performed for strain selection and therefore was performed once as a screen.

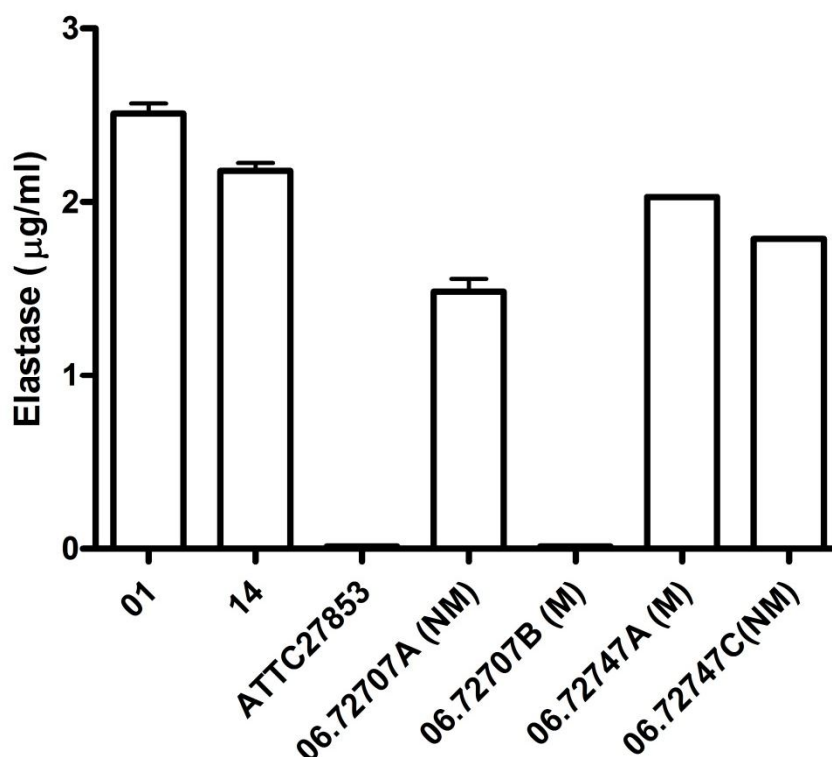


Figure 3.7. Elastase production of selected strains of *Pseudomonas aeruginosa*.

P. aeruginosa elastase production is variable. Seven strains of *P. aeruginosa* were standardised to 1×10^6 cells/ml in RPMI-1640 media, with 500µl added to a 24-well plate for 24 h before quantification of elastase. A significant difference ($p < 0.001$) was found to occur between the various strains. The bars and whisker represent the mean plus standard deviation of the mean. $n=1$.

3.3.2.4. *Pseudomonas aeruginosa* mutants deficient in elastase production were not detected by the ECR assay

This study aimed to verify that the ECR assay successfully measured elastase production in *P. aeruginosa*. Mutants with deletions in the genes controlling the expression of elastase (PA01 $\Delta LasI$ and PA01 $\Delta LasR$) were selected as controls. PA01 $\Delta LasI$ is unable to produce homoserine lactones and PA01 $\Delta LasR$ is able to produce homoserine lactones but unable to respond to them. These were compared against the wild type PA01 strain to show that the assay did not detect elastase in strains deficient in elastase production (Figure 3.8).

Elastase production of PA01 wild type in culture was significantly higher than that of PA01 $\Delta LasI$ and PA01 $\Delta LasR$ (ANOVA and the Bonferroni post-test $p < 0.001$) from a mean production of 1.12 $\mu\text{g/ml}$ elastase to 0.12 $\mu\text{g/ml}$ elastase (89.3% decrease) and 0.10 $\mu\text{g/ml}$ elastase (91.1% decrease), respectively. These results indicated that the elastase production of PA01 in culture was considerably greater than that of either elastase-deficient mutant as measured by the ECR assay, verifying the validity of the assay.

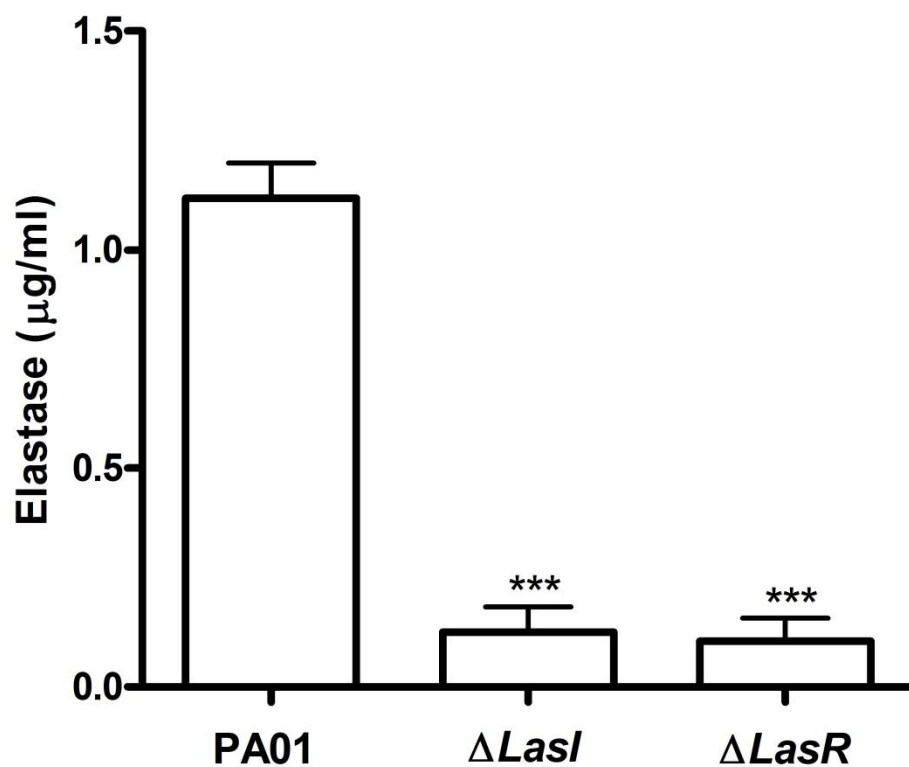


Figure 3.8. Elastase-deficient PA01 strains are not detected by the ECR assay.

PA01 and two respective quorum sensing knockout mutants, $\Delta LasI$ and $\Delta LasR$, were adjusted to 1×10^6 cells/ml in RPMI-1640. Cells were incubated for 24 h in a 24 well plate at 500 μl per well in RPMI-1640 before elastase quantification by the ECR assay. A significantly higher level of elastase ($p < 0.001$) was observed in PA01 in comparison to both quorum sensing mutants. Each experiment was carried on three separate occasions and performed triplicate, with bars and whisker representing the mean plus standard deviation of the mean. $n=3$.

3.3.3. Elastase production of bacteria in response to 24 hour *Aspergillus fumigatus*

These studies were designed to test the hypothesis that the presence of *A. fumigatus* 24 h biofilm would lead to a change in virulence expression in *P. aeruginosa* during co-culture of the two organisms. Moreover, a secondary relevant bacterial pathogen of the CF lung, *Burkholderia cenocepacia*, was also examined. In order to test this hypothesis, a series of experiments were carried out in which two *P. aeruginosa* wild-type strains (PA01 and PA14), and one *B. cenocepacia* wild-type strain (K562), were incubated with 24 h live or dead *A. fumigatus* biofilms and the quantities of virulence factor elastase produced were measured.

3.3.3.1. Elastase production by *Pseudomonas aeruginosa* in response to *Aspergillus fumigatus* biofilm

Elastase production of wild-type *P. aeruginosa* was measured in response to both live and dead 24 h Af293 biofilm in comparison with untreated control, following 24 h co-incubation.

Elastase production of PA01 culture (Figure 3.9A) was significantly increased in the presence of dead 24 h Af293 biofilm (ANOVA and the Dunnett post-test $p < 0.001$) as with PA14, increasing from a mean production of 0.77 $\mu\text{g/ml}$ elastase to 1.77 $\mu\text{g/ml}$ elastase, showing a 127.9% increase. However, no significant difference was observed for PA01 in culture in the presence of live 24h Af293 biofilm (ANOVA and the Dunnett post-test $p > 0.05$) with the treated PA01 producing a mean production of 0.2 $\mu\text{g/ml}$ elastase in comparison to the control producing 0.77 $\mu\text{g/ml}$ elastase, a decrease of 74.5%.

Elastase production of PA14 culture (Figure 3.9) was significantly increased in the presence of dead 24 h Af293 biofilm (ANOVA and the Dunnett post-test $p < 0.01$) from a mean production of 1.03 $\mu\text{g/ml}$ elastase to 1.67 $\mu\text{g/ml}$ elastase, showing a 61% increase in comparison with the control. In addition to this, elastase production of PA14 was significantly decreased in the presence of live 24 h Af293 biofilm (ANOVA and the Dunnett post-test $p < 0.01$) from 1.03 $\mu\text{g/ml}$ elastase to 0.20 $\mu\text{g/ml}$ elastase, an 80.54% decrease in comparison with the control. These results indicated that the elastase production of PA14 in culture

varies due to the presence of both live and dead 24 h Af293 biofilm. The trend of dead 24 h Af293 biofilm being associated with an increase in elastase is maintained between PA14 and PA01, whereas a decrease in elastase production in response to live 24h Af293 biofilm is only observed in PA14, not PA01.

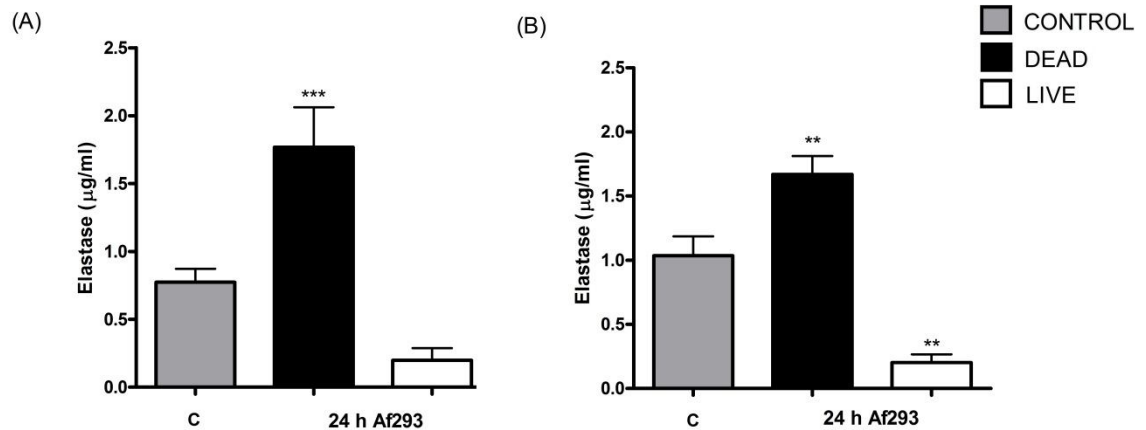


Figure 3.9. Elastase production of *Pseudomonas aeruginosa* in response to 24 hour *Aspergillus fumigatus* biofilm.

(A) PA01 and (B) PA14 were grown at 1×10^6 cells/ml in a final volume of 500 µl in RPMI-1640 media in a 24-well plate in the presence of live or dead 24 h Af293 biofilm, and a control containing no Af293 biofilm, for 24 h before quantification of elastase. Each experiment was carried on three separate occasions and performed in triplicate, with bars and whisker representing the mean plus standard deviation of the mean. $n=3$.

3.3.3.2. Elastase production of wild type *Burkholderia cenocepacia* in response to 24 hour *Aspergillus fumigatus* biofilm

Although *P. aeruginosa* is the most prominent bacterium associated with CF, multiple other species are also associated with the disease, including *B. cenocepacia* (Govan and Deretic 1996). In order to determine whether the effects of Af293 on *P. aeruginosa* were unique to that organism or also occurred in other relevant bacteria, the effect of 24 h Af293 biofilm on *B. cenocepacia* K562 was determined (Figure 3.10).

Elastase production of *B. cenocepacia* K562 was not significantly increased in the presence of dead 24h Af293 biofilm (ANOVA and the Dunnett post-test $p>0.05$) unlike the *P. aeruginosa* strains tested, where elastase was increased from a mean production of 3.71 µg/ml to 5.7 µg/ml, a 53.2% increase. A significant

decrease was observed for *B. cenocepacia* K562 in culture in the presence of live 24h Af293 biofilm (ANOVA and the Dunnett post-test $p < 0.01$) with the treated *B. cenocepacia* K562 producing 0.11 $\mu\text{g/ml}$ elastase in comparison to the control producing 3.71 $\mu\text{g/ml}$, a 97% decrease. These results indicated that *B. cenocepacia* did not produce an increased level of elastase in response to dead 24h Af293 biofilm, in contrast with both *P. aeruginosa* strains treated, but a decrease in elastase is observed in response to dead 24h Af293 biofilm as with PA01.

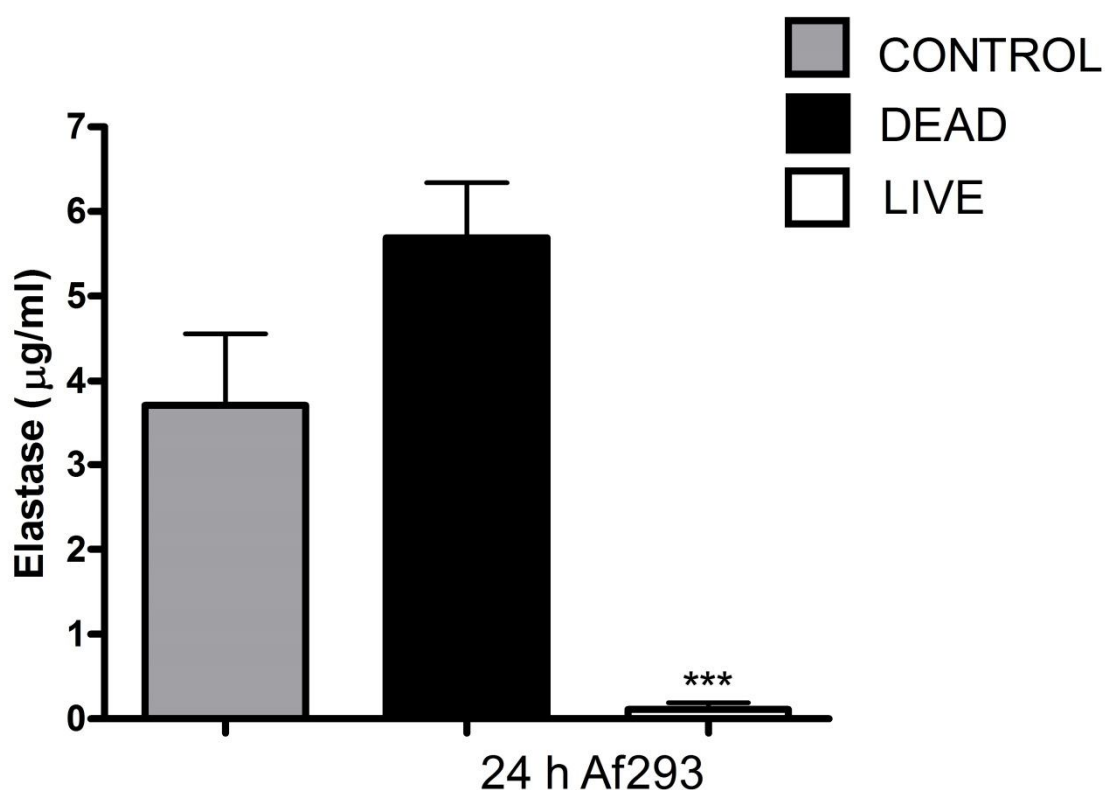


Figure 3.10. Elastase production of *Burkholderia cenocepacia* K562 in response to 24 hour *Aspergillus fumigatus* biofilm.

B. cenocepacia K562 was grown at 1×10^6 cells/ml in a final volume of 500 μl in RPMI-1640 media in a 24-well plate in the presence of live or dead 24 h Af293 biofilm, and a control containing no Af293 biofilm, for 24 h before quantification of elastase. Each experiment was carried on three separate occasions and performed in triplicate, with bars and whisker representing the mean plus standard deviation of the mean. $n=3$.

3.3.3.3. Comparison of elastase production from mucoid and non-mucoid *Pseudomonas aeruginosa* in the presence and absence of 24 hour *Aspergillus fumigatus* biofilm

A common phenotypic change of *P. aeruginosa* within the CF lung is that of the non-mucoid phenotype to the mucoid (Govan and Deretic 1996). That is, the bacteria over-express alginate during the chronic stage of respiratory infection in CF which increases their pathogenic potential. A mucoid and non-mucoid clinical isolate, PA 06.72747A and PA 06.72747C respectively, were tested in order to compare the two phenotypes in regards to elastase production in the presence of live and dead 24 h Af293 biofilm (Fig 3.11).

Elastase production of PA 06.72747A (M) was significantly increased in the presence of dead 24 h Af293 biofilm (ANOVA and the Dunnett post-test $p<0.05$) from a mean production of 1.58 µg/ml elastase to 3.09 µg/ml elastase, showing a 95.4% increase in comparison with the control. In addition, elastase production of PA 06.72747A (M) was significantly decreased in the presence of live Af293 biofilm (ANOVA and the Dunnett post-test $p<0.05$) from 1.58 µg/ml elastase to 0.14 µg/ml elastase, a 91.3% decrease in comparison with the control. Elastase production of PA 06.72747C (NM) was significantly increased by the presence of dead 24 h Af293 biofilm (ANOVA and the Dunnett post-test $p<0.001$) from a mean production of 0.73 µg/ml elastase to 1.83 µg/ml elastase, showing a 150.5% increase in comparison with the control. Elastase production of PA 06.72747C (NM) culture showed a significant decrease in the presence of live Af293 biofilm (ANOVA and the Dunnett post-test $p<0.01$) from 0.73 µg/ml elastase to 0.17 µg/ml elastase, a 76.7% decrease in comparison with the control. These results indicated that elastase production of both mucoid and non-mucoid *P. aeruginosa* were significantly affected by the presence of both live and dead 24 h Af293 biofilms. This was more significant for non-mucoid strains. Due to the greater relevance of mucoid *P. aeruginosa* to the CF lung in comparison with non-mucoid *P. aeruginosa*, this study did not reveal a greater risk of increased elastase production in strains of greater clinical relevance.

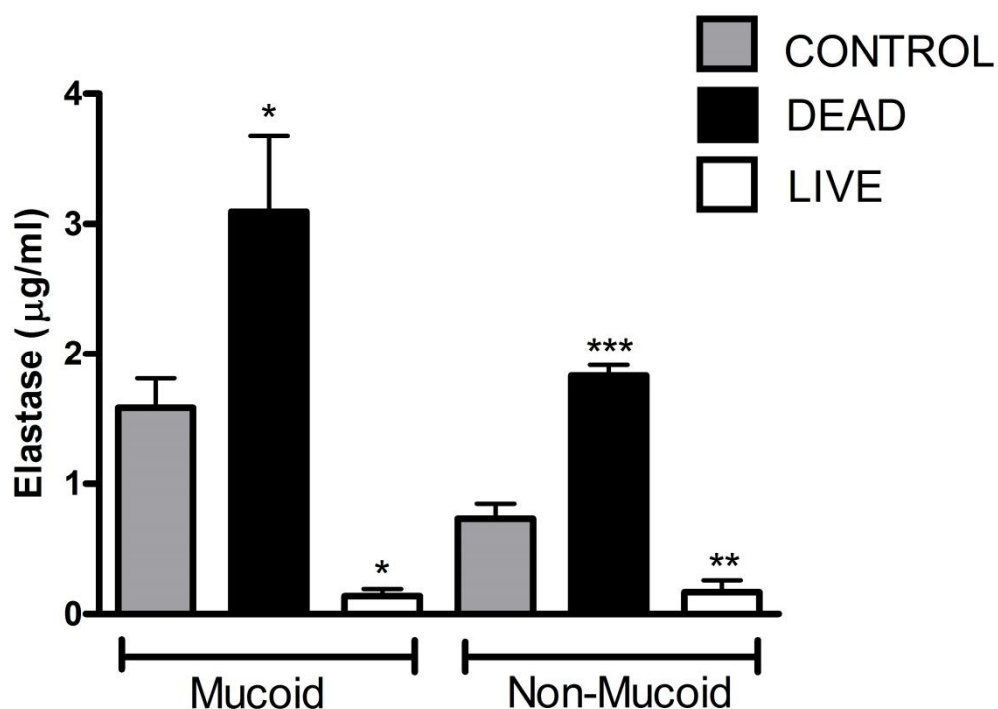


Figure 3.11. Comparison of the elastase production of mucoid and non-mucoid *Pseudomonas aeruginosa* in the presence and absence of *Aspergillus fumigatus* biofilm.

PA 06.72747A (mucoid) and PA 06.72747C (non-mucoid) were grown at 1×10^6 cells/ml in a final volume of 500 µl in RPMI-1640 media in a 24-well plate in the presence of live 24 h Af293 biofilm or dead 24 h Af293 biofilm, and a control containing no Af293 biofilm, for 24 h before quantification of elastase. Each experiment was carried on three separate occasions and performed triplicate, with bars and whisker representing the mean plus standard deviation of the mean. $n=3$.

3.3.4. Effect of various stages of *Aspergillus fumigatus* biofilm growth on the elastase production of *Pseudomonas aeruginosa*

A. fumigatus has multiple distinct phases of biofilm development. Four time points were selected to correspond with these stages - 8 h (germlings), 12 h (monolayer of proliferating mycelia) and 24 h and 48 h (biofilm, with densely intertwined hyphae) (Rajendran *et al.*, 2011). As it is unlikely that *P. aeruginosa* would simply encounter *A. fumigatus* at precisely 24 h, this study allowed for a broader range of *A. fumigatus* biofilms to be tested in order to reveal possible variations in *P. aeruginosa* elastase production in response to *A. fumigatus*. Type strains PA01 and PA14 were incubated in culture with all 4 Af293 time

points and a control containing no Af293 biofilm for 24 h before elastase quantification.

Elastase production of PA01 culture (Figure 3.12) was significantly increased in the presence of dead 8 h, 12 h and 24 h Af293 biofilm (ANOVA and the Dunnett post-test $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). A mean production of 0.51 $\mu\text{g/ml}$ elastase for PA01 alone rose to 1.68 $\mu\text{g/ml}$ elastase in the presence of dead 12 h Af293, showing a 171.3% increase, and to 1.78 $\mu\text{g/ml}$ elastase in the presence of dead 24 h Af293, showing a 246.9% increase. Elastase production of PA01 in culture was not significantly affected (ANOVA and the Dunnett post-test $p > 0.05$) by the presence of dead 8 h Af293 or 48 h Af293, with resulting mean productions of elastase of 1.38 $\mu\text{g/ml}$ (171.2% increase) and 0.94 $\mu\text{g/ml}$ (83.9% increase) respectively.

Moreover, the presence of live 8, 12, 24 and 48 h live Af293 led to no significant difference (ANOVA and the Dunnett post-test $p > 0.05$) in elastase production of PA01 in culture, resulting in mean elastase production of 0.29 $\mu\text{g/ml}$ (42.2% decrease), 0.19 $\mu\text{g/ml}$ (62.1% decrease), 0.20 $\mu\text{g/ml}$ (61.1% decrease) and 0.22 $\mu\text{g/ml}$ (56.9% decrease), respectively, in comparison with the control.

In contrast with that of PA01 culture, elastase production of PA14 culture was significantly increased in the presence of all four dead Af293 biofilm phases. The level of statistical significance varied over 8, 12, 24 and 48 h (ANOVA and the Dunnett post-test $p < 0.05$, $p < 0.001$, $p < 0.001$ and $p < 0.05$ respectively). The mean production for PA14 alone was 0.63 $\mu\text{g/ml}$ elastase, which rose to 1.68 $\mu\text{g/ml}$ elastase in the presence of dead 8 h Af293, (202.4% increase), to 2.48 $\mu\text{g/ml}$ elastase in the presence of dead 12 h Af293 (293.8% increase), to 2.53 $\mu\text{g/ml}$ elastase in the presence of dead 24 h Af293 (302.5% increase) and to 1.87 $\mu\text{g/ml}$ in the presence of dead 48 h Af293 (197.7% increase).

The presence of live 8, 12, 24 and 48 h live Af293 had no significant difference (ANOVA and the Dunnett post-test $p > 0.05$) in elastase production of PA14 in culture, resulting in mean elastase production of 0.16 $\mu\text{g/ml}$ (74.1% decrease), 0.36 $\mu\text{g/ml}$ (42.6% decrease), 0.22 $\mu\text{g/ml}$ (65.7% decrease) and 0.17 $\mu\text{g/ml}$ (27.8%), respectively, in comparison with the control.

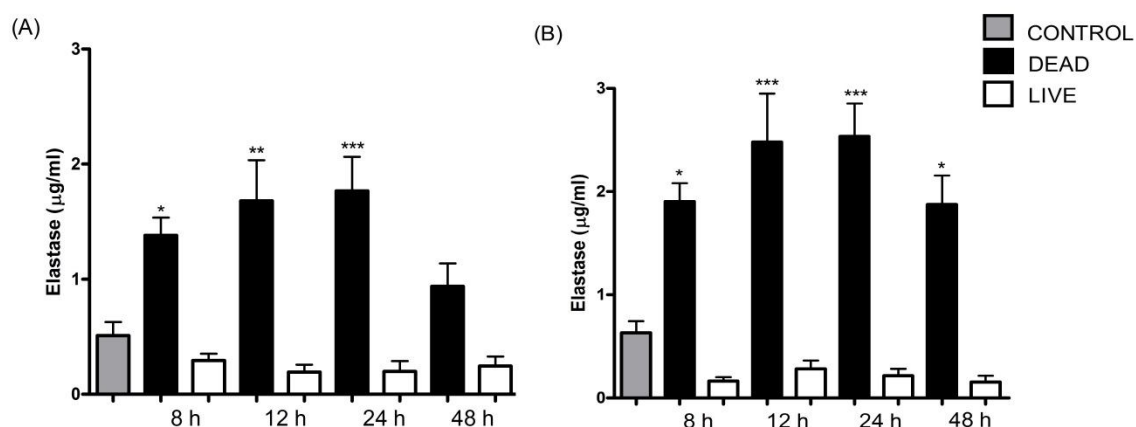


Figure 3.12. Effect of various phases of *Aspergillus fumigatus* biofilm growth on the elastase production of *Pseudomonas aeruginosa*.

(A) PA01 and (B) PA14 were grown at 1×10^6 cells/ml in a final volume of 500 µl in RPMI-1640 media in a 24-well plate in the presence of live or dead 8 h, 12 h, 24 h or 48 h Af293 and a control, for 24 h before quantification of elastase. Each experiment was carried on three separate occasions and performed triplicate, with bars and whisker representing the mean plus standard deviation of the mean. $n=3$.

3.3.5. Effect of an inorganic substrate (latex beads) on elastase production by *Pseudomonas aeruginosa*

A control experiment was set up using an inorganic substrate (0.8 µm latex beads, Sigma) to test whether the presence of a physical scaffold led to an alteration in elastase production by *P. aeruginosa*. 10 µl of latex beads were diluted two-fold to give a range of concentrations for testing. Two strains PA01 and PA14 were used in these experiments. Elastase production by PA01 and PA14 (Figure 3.13) was not significantly affected by the presence of latex beads (ANOVA $p > 0.05$). These results indicated that the increase in elastase activity observed previously in response to dead Af293 biofilm was not simply due to the presence of a physical structure in the *P. aeruginosa* culture.

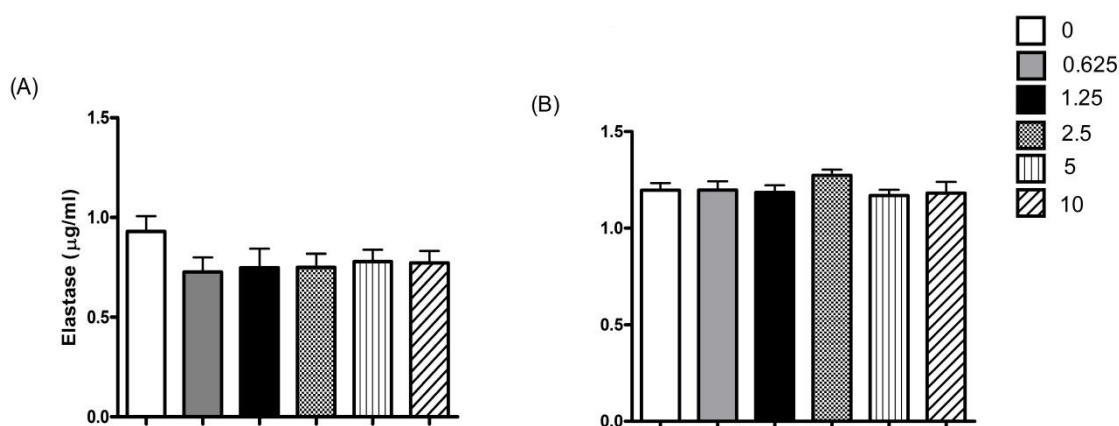


Figure 3.13. Effect of an inorganic substrate (latex beads) on elastase production by *Pseudomonas aeruginosa*.

Both (A) PA01 and (B) PA14 were grown at 1×10^6 cells/ml in a final volume of 500 µl of inRPMI-1640 media in a 24-well plate in the presence and absence of two fold dilutions of latex beads (starting with 1/50 dilution of beads from stock, equivalent to 10 µl of latex beads) for 24 h before quantification of elastase. Each experiment was carried on three separate occasions and performed triplicate, with bars and whisker representing the mean plus standard deviation of the mean. $n=3$.

3.3.6. Effect of disrupting *Aspergillus fumigatus* biofilm structure on *Pseudomonas aeruginosa* elastase production

Given the conclusion that dead Af293 biofilm induces a significant increase in elastase production by *P. aeruginosa*, the question of why this occurs must be asked. This study tested if the physical structure of the biofilm was responsible. To test this, dead 24 h Af293 biofilms were disrupted as described in Chapter 2.1.2.3 and incubated with PA01 over 24 h (Figure 3.14).

Elastase production of PA01 was significantly increased in the presence of intact dead 24 h Af293 biofilm (ANOVA and the Bonferroni post-test $p < 0.001$) from a mean production of 0.23 µg/ml elastase to 0.63 µg/ml elastase, showing a 177.5% increase in comparison with the control. In contrast to this, elastase production of PA01 was not significantly affected by the presence of disrupted dead 24 h Af293 biofilm (ANOVA and the Bonferroni post-test $p > 0.05$) from 0.23 µg/ml elastase to 0.26 µg/ml elastase, a 14.9% in comparison with the control. These

results indicated that the structure of dead 24 h Af293 biofilm was essential to the increase in elastolytic activity observed in PA01.

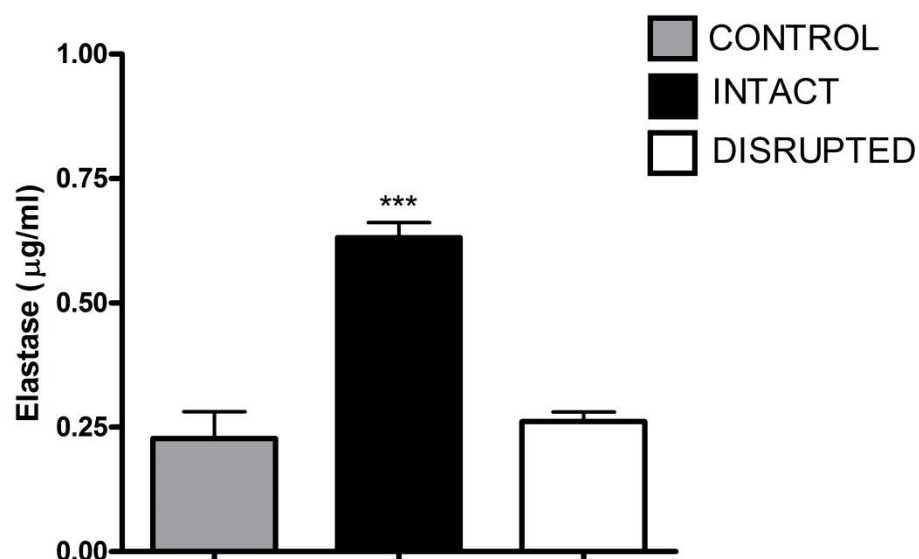


Figure 3.14. Effect of disrupting *Aspergillus fumigatus* biofilm structure on *Pseudomonas aeruginosa* elastase production.

PA01 was grown at 1×10^6 cells/ml in a final volume of 500µl in RPMI-1640 media in a 24-well plate in the presence of intact dead 24 h Af293 biofilm or at 2×10^6 cells/ml in a final volume of 250µl in RPMI-1640 media with disrupted dead 24h Af293 biofilm at a final volume of 250µl in RPMI-1640 media, and a control containing no Af293 biofilm, for 24 h before quantification of elastase. Each experiment was carried on three separate occasions and performed triplicate, with bars and whisker representing the mean plus standard deviation of the mean. $n=3$.

3.4. Expression of *Pseudomonas aeruginosa* *LasB* in response to *Aspergillus fumigatus*

3.4.1. Primer validation

In order to validate the primers selected for this study, a serial tenfold dilution of cDNA extracted from PA01 was used to demonstrate that primer binding increased as concentration of cDNA increased (Figure 3.15). The protocol for this was taken from the paper in which the primers were originally described (Lee *et al.*, 2011). Primer efficiency was calculated as percentage efficiency, showing that *rpoD* (encoding for an RNA polymerase) was 96% efficient and *LasB* (encoding elastase) was 92% efficient. Primer efficiency is regarded as acceptable when it falls between 90% and 110% (Taylor *et al.*, 2010). Therefore, the primers efficiently amplified cDNA, meaning they were sufficient for use in these studies.

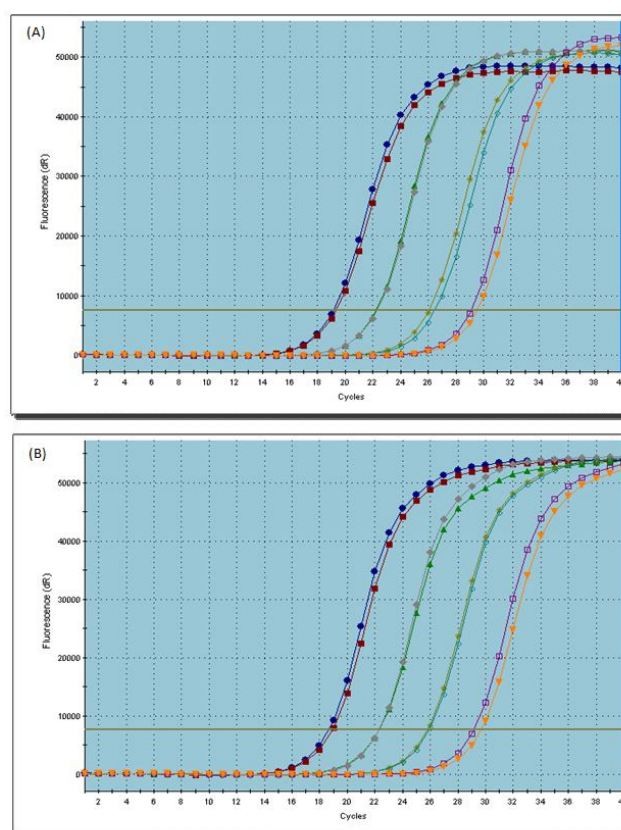


Figure 3.15. Validation of *rpoD* and primers.

Tenfold serial dilutions of PA01 cDNA (2 ng/μl, 0.2 ng/μl, 0.02 ng/μl and 0.002 ng/μl) were used for assessing primers for (A) *rpoD* and (B) *LasB*, in duplicate. $n=1$.

3.4.2. Gene expression of *Pseudomonas aeruginosa* LasB in response to *Aspergillus fumigatus* biofilm

Real-time PCR was carried out to assess the levels of *LasB* gene expression in PA01 in the presence and absence of dead 24 h Af293 biofilm at various time points (1, 4, 8 and 24 h). Gene regulation was standardised to housekeeping gene *rpoD*. Fold change in the presence of dead Af293 biofilm was determined individually for each time point (Figure 3.16). Fold change of *LasB* expression in PA01 grown with dead 24 h Af293 biofilm varies as follows: at 1 h there was a 0.45 fold decrease, at 4 h there was a 3.66 fold increase, at 8 h there was a 0.58 fold decrease, and at 24 h there was a 0.23 fold decrease. For statistical analysis, the fold change data was subjected to a natural log transformation ($\ln(+k)$) before analysis by t test. No significant difference ($p > 0.05$) was found in gene expression of *LasB* relative to *rpoD* at any time point.

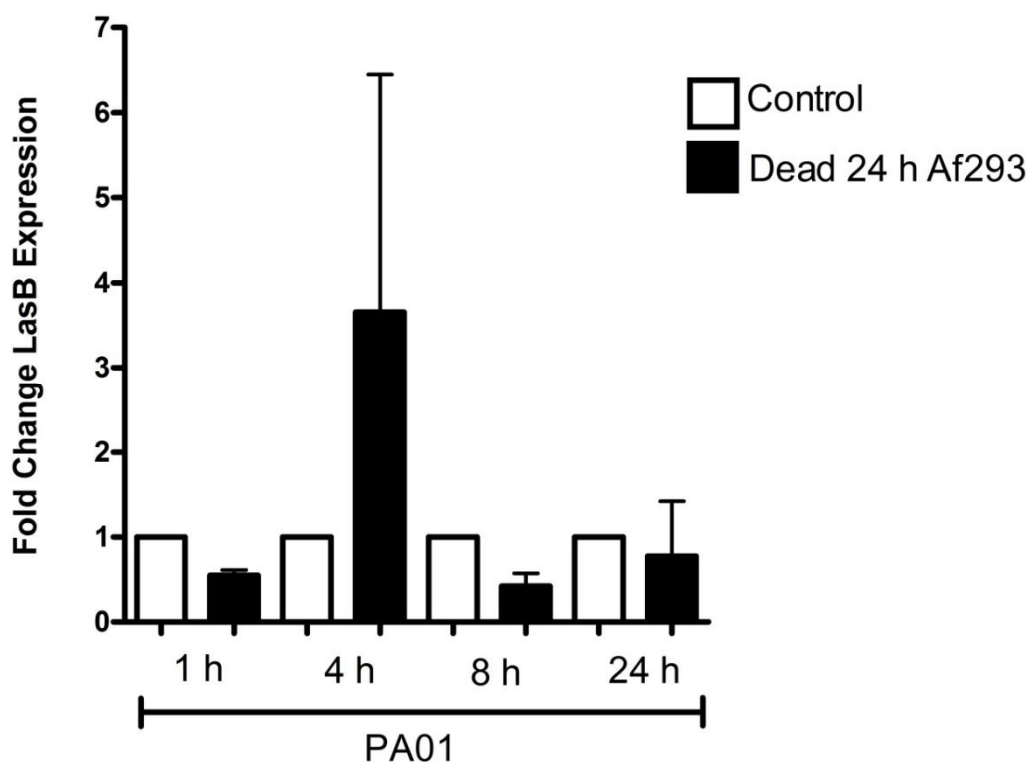


Figure 3.16. *Pseudomonas aeruginosa* LasB expression varies over time in response to dead 24 hour *Aspergillus fumigatus* biofilm.

LasB gene regulation in PA01 cells stimulated with dead 24 h Af293 biofilm was assessed through real-time PCR using extracted DNA from *P. aeruginosa*. Fold change was calculated in relation to a PA01 control using *rpoD* as a housekeeping gene. Fold change for PA01 in the presence of dead Af293 at each time point is determined in relation to the respective expression at that time point. Experiments were carried out a total of 3 times, in duplicate. n=3.

3.5. Effect of *Pseudomonas aeruginosa* and *Aspergillus fumigatus* on a human lung cell line

3.5.1. Effect of *Pseudomonas aeruginosa* and *Aspergillus fumigatus* supernatants on A549 cells

As *P. aeruginosa* and *A. fumigatus* are known pathogens of the CF lung, this study sought to determine how supernatants from both organisms alone and from co-cultures affected an epithelial cell line taken from the human lung (A549) as a representation of how these species might affect the CF lung *in vivo*.

3.5.1.1. Effect of *Pseudomonas aeruginosa*, *Aspergillus fumigatus* and combinational supernatants on A549 cell viability

Supernatants were collected from the following cultures following 24 h incubation: PA14; live 24 h Af293 biofilm; dead 24 h Af293 biofilm; PA14 co-cultured with live 24 h Af293 biofilm; and PA14 co-cultured with dead 24 h Af293 biofilm. Uncultured 0 h Af293 conidia were also used as a control. A549 cells were treated with microbial supernatants for 24 h as described in Chapter 2.4.5.1., prior to cell viability testing using alamarBlue. For statistical analysis, the % reduction data was subjected to a natural log transformation ($\ln(+k)$) before the following analysis: PA14 was compared with PA14 plus live Af293 and with PA14 with dead Af293 using one-way ANOVA and the Dunnett post test. In addition comparisons were made between live and dead Af293, between live Af293 and live Af293 plus PA14 and between dead Af293 and dead Af293 plus PA14 using a the t test with a Bonferroni correction.

In comparison with the media control, the % reduction of alamarBlue observed for the supernatants tested were as follows: PA14 (91.77% reduction); 0 h Af293 conidia (14.3% reduction); live 24 h Af293 biofilm (11.99% reduction); dead 24 h Af293 biofilm (93.87% reduction); PA14 co-cultured with live 24 h Af293 biofilm (12.6% reduction); and PA14 co-cultured with dead 24 h Af293 biofilm (1.65% increase).

PA14 resulted in a considerable reduction of alamarBlue by A549 cells in comparison with media control (ANOVA and the Dunnett post-test $p < 0.01$). The use of any supernatants containing live Af293 biofilm (0 h conidia or 24 h

biofilm, with or without PA14) resulted in a considerable % reduction of alamarBlue (ANOVA and the Dunnett post-test $p < 0.001$), demonstrating the highly cytotoxic effect of live Af293 on A549 cells. In contrast to this, supernatants containing dead 24 h Af293 biofilm (alone, and with PA14) showed no considerable reduction in % reduction of alamarBlue (ANOVA and the Dunnett post-test $p > 0.05$). No difference was found to occur in A549 cell viability between dead 24 h Af293 biofilm and 24 h dead Af293 biofilm co-cultured with PA14 (ANOVA and the Dunnett post-test $p < 0.05$). A highly significant difference occurred in A549 cell viability between live and dead 24 h Af293 with live Af293 causing a considerable loss in cell viability (t test with Bonferonni correction, $p < 0.001$).

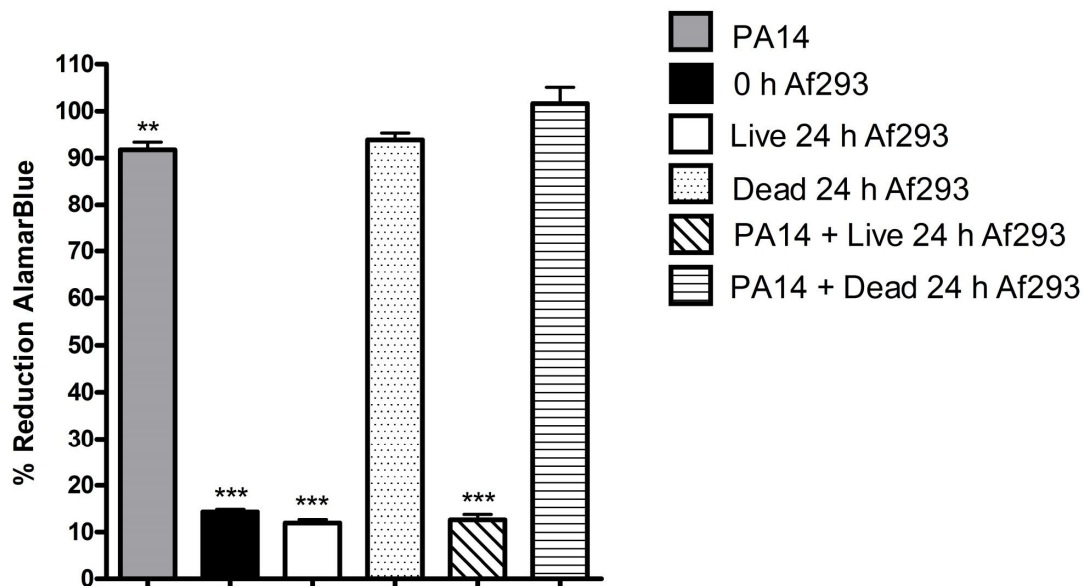


Figure 3.17. Effect of *Pseudomonas aeruginosa*, *Aspergillus fumigatus* and combinational supernatants on A549 cell viability.

Supernatants taken from PA14, Af293 and co-cultures of the two species result in various effects on the % reduction of AlamarBlue by A549 cells. A549 alveolar lung epithelial cells were cultured in 24 well tissue culture plates. Cells were stimulated with supernatants from the following 24 h cultures: PA14; 0 h Af293 conidia; live 24 h Af293 biofilm; dead 24 h Af293 biofilm; PA14 co-cultured with live 24 h Af293 biofilm; and PA14 co-cultured with dead 24 h Af293 biofilm. Percentage reduction of alamarBlue was calculated relative to a media only control. Each experiment was carried on three separate occasions and performed in triplicate, with bars and whiskers representing the mean plus standard deviation of the mean. $n=3$.

3.5.1.2. Light microscopy of the effect of *Pseudomonas aeruginosa*, *Aspergillus fumigatus* and combinational supernatants on A549 cells

Light microscopy was utilised to examine how the morphology of A549 cells varied after treatment with *P. aeruginosa*, 0 h conidia, both live and dead 24 h *A. fumigatus*, and combinational supernatants in comparison with an untreated control (Figure 3.18).

In the untreated control, A549 cells remain adherent to the plastic surface of the well. Most cells show a shape characteristic of A549 cells (as shown) although some showed a more rounded circular morphology. Treatment with PA14 alone resulted in a mixture of adherent cells, rounded cells and rounded cells which had detached from the plastic surface. When supernatants taken from PA14 co-cultured with dead 24 h Af293 biofilm (which had been shown to cause little difference to the morphology of cells in comparison with the control), fewer adherent cells were noted in comparison with supernatants taken from PA14 culture alone, with almost all cells being detached and rounded. Supernatants from cultures containing live Af293 (0 h Af293, live 24 h Af293 biofilm and PA14 co-cultured with live 24 h Af293 biofilm) all displayed clear cell blebbing and cell damage (Fackler and Grosse 2008), demonstrating that live Af293 had a strong negative effect on cell morphology.

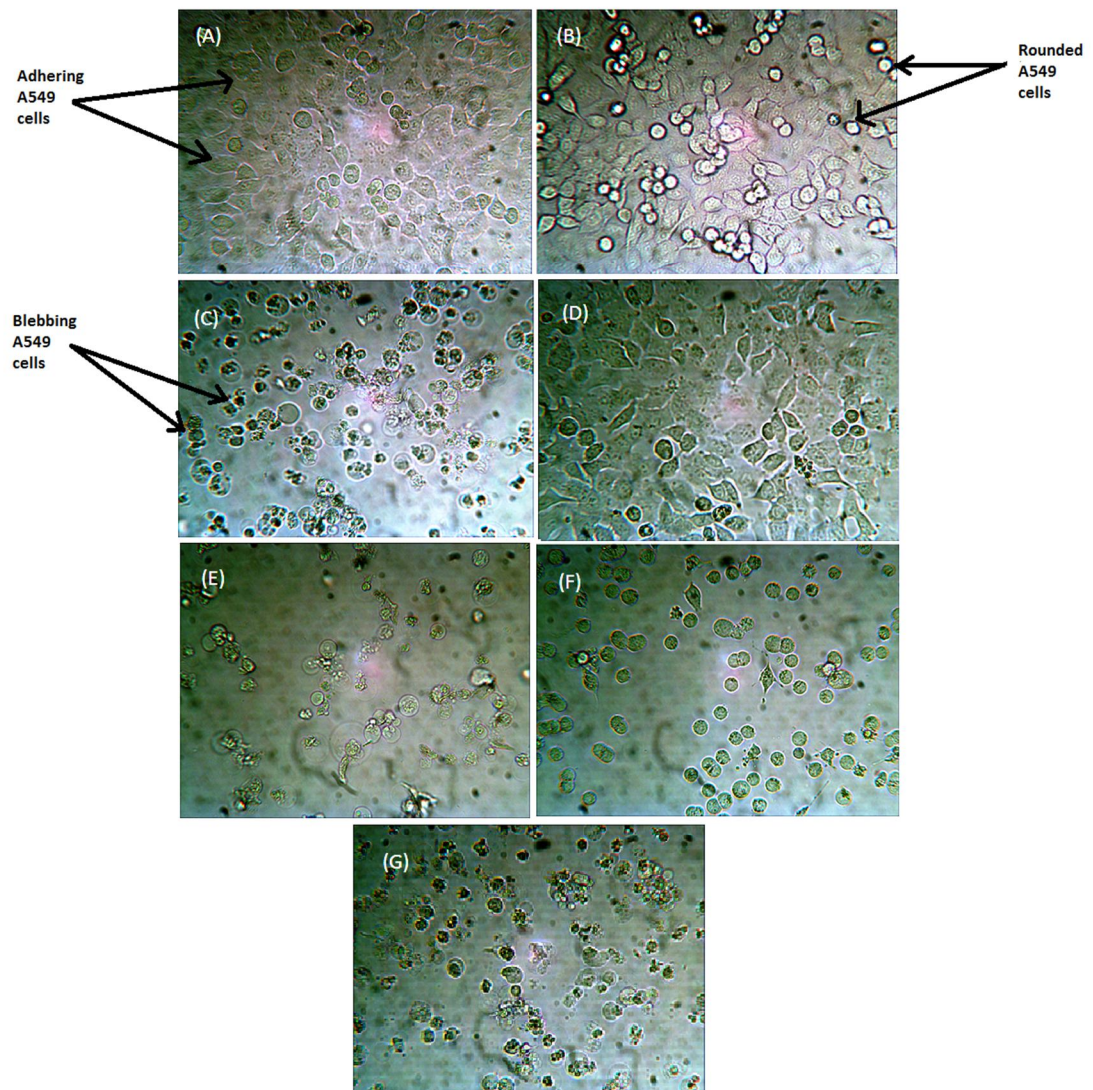


Figure 3.18. Light microscopy of the effect of supernatants from *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, and combinational co-cultures on the A549 cell line.

Light microscopy of a field within the well of a 24-well plate containing A549 cells treated with different supernatants at $\times 25$ magnification. Supernatants tested were taken from 24 h cultures of: (A) RPMI-1640 control; (B) PA14; (C) 0 h Af293 conidia; (D) dead Af293 biofilm; (E) live Af293 biofilm; (F) PA14 incubated with dead 24 h Af293; (G) PA14 incubated with live Af293 biofilm for 24 h. Each photograph is taken from one representative experiment. $n=1$.

3.5.2. Effect of elastase on the A549 cell line

Previous studies used the surrogate virulence factor elastase from *P. aeruginosa* as an indicator of virulence alterations in *P. aeruginosa* due to the presence of *A. fumigatus*. By demonstrating whether or not elastase had a notable effect on A549 lung cells, both in regards to cell viability and morphology, this study aimed to establish the effect of the surrogate virulence factor on cells relevant to respiratory disease.

3.5.2.1. Effect of elastase on A549 cell viability

This study aimed to examine how elastase affected A549 cell viability, as measured by the alamarBlue assay. Several concentrations of elastase (0.5, 4, 16 and 128 µg/ml) were incubated with A549 cells for 24 h before cell viability was measured (Figure 3.19). For statistical analysis, the % reduction data was subjected to a natural log transformation ($\ln(+k)$) before analysis by one-way ANOVA and the Bonferroni post-test. No significant difference (ANOVA and the Bonferroni post-test $p>0.05$) was found with concentrations of elastase.

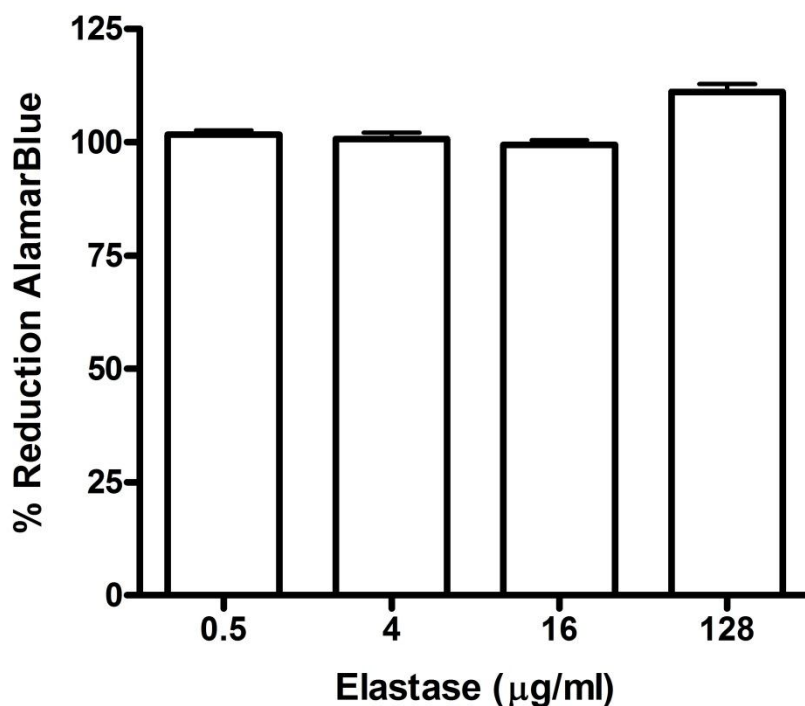


Figure 3.19. Effect of elastase on A549 cell viability.

A549 alveolar lung epithelial cells were cultured in 24 well tissue culture plates for 24 h at 37°C in 5% CO₂/ air atmosphere. Cells were stimulated with various concentrations of elastase (0.5, 4, 16, 128µg/ml), in relation to a media control. Following 24 h incubation, alamarBlue was added and absorbance readings were taken after 3 h incubation. Each experiment was carried on three separate occasions and performed triplicate, with bars and whisker representing the mean plus standard deviation of the mean. n=3.

3.5.2.2. Light Microscopy of effect of elastase on A549 cells

Light microscopy was utilised to examine how the appearance of A549 cells varied after treatment (Figure 3.20) in comparison with a media control. It was shown that little difference was found at concentrations as high as 16 µg/ml, with cell morphology remaining very similar to that of the media control, with the exception of 4 µg/ml elastase treatment in which some cell rounding was noted. However, at 128 µg/ml the vast majority of A549 cells became rounded and detached from the plastic surface of the 24-well plate.

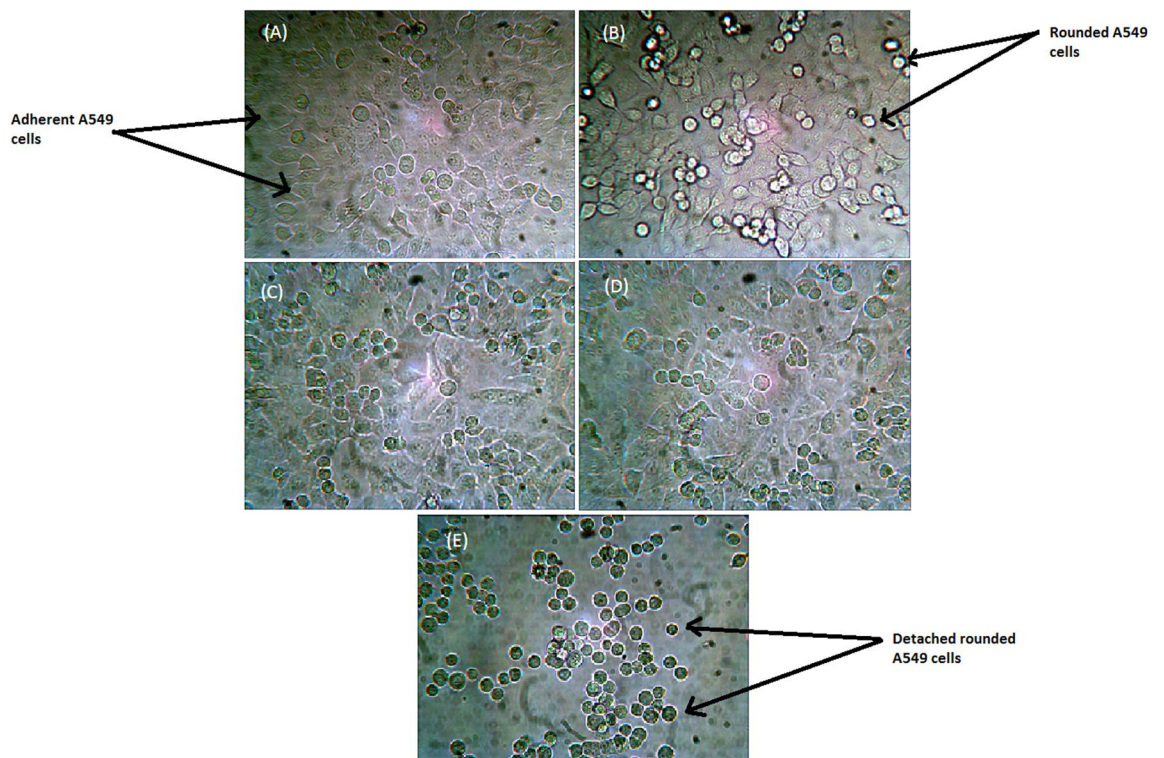


Figure 3.20. Effect of elastase on A549 cell morphology.

Light microscopy of a field within the well of a 24-well plate containing A549 cells treated with different concentrations of pancreatic porcine elastase at $\times 25$ magnification. The concentrations tested were: (A) RPMI-1640 control; (B) $0.5 \mu\text{g/ml}$ elastase; (C) $4 \mu\text{g/ml}$ elastase; (D) $16 \mu\text{g/ml}$ elastase; (E) $128 \mu\text{g/ml}$ elastase. Each photograph is taken from one representative experiment. $n=1$.

3.6. Wax Worm Model of *Pseudomonas* and *Aspergillus* co-infection

3.6.1. Survival rates of *Galleria mellonella* injected with various concentrations of *P. aeruginosa*

These studies aimed to establish the killing effect of various concentrations of *P. aeruginosa* type strain PA14 on *G. mellonella* wax worms. This was to establish if a dose dependent response existed, and if so which concentration resulted in mortality of the wax worms at a rate allowing for an increase in virulence when co-incubated with dead Af293 germlings to be observable due to a comparative decrease in survival rates (Figure 3.21).

It was shown that there was a significant difference (Logrank test $p < 0.001$) between the survival rates of worms injected with PA14 of various concentrations. The median survival for injected worms was as follows: 1×10^5 cells/ml (12 h); 1×10^4 and 1×10^3 cells/ml (18 h); 1×10^2 cells/ml (24 h); and 1×10^1 cells/ml (42 h). 18 h was decided as the optimum median survival rate, and 1×10^3 cells/ml was decided on as, despite the median remaining the same, more worms died at an earlier rate for 1×10^4 cells/ml.

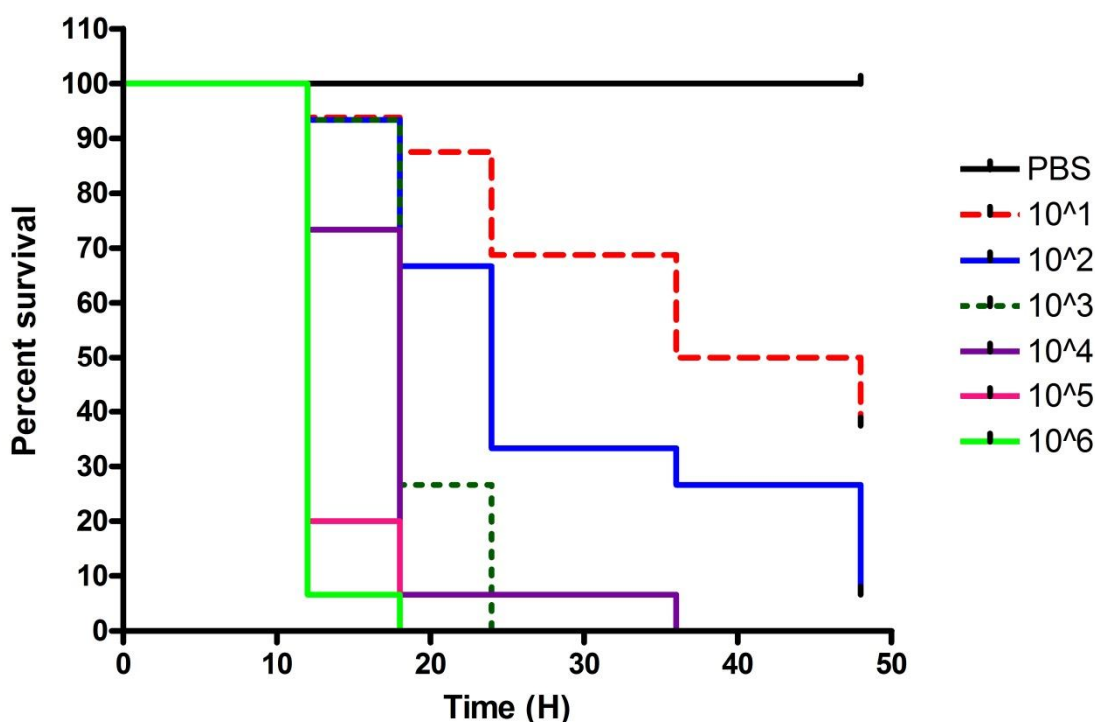


Figure 3.21. Survival rates of *Galleria mellonella* injected with various concentrations of *Pseudomonas aeruginosa*.

Wax worm larvae were inoculated with 10µl of serial dilutions (10^5 , 10^4 , 10^3 , 10^2 and 10^1 cells/ml) of strain PA14 suspended in PBS, as well as a PBS control. Survival was monitored for 48 h. A dose-dependent response was found. Kaplan-Meier survival curves were determined for three independent experiments each using five worms per condition. $n=3$.

3.6.2. Survival rates of *G. mellonella* injected with killed *A. fumigatus* germlings

The aim of this experiment was to demonstrate that dead *A. fumigatus* caused no significant effect on the survival of wax worms. This would therefore mean that if a combination of dead *A. fumigatus* and live *P. aeruginosa* led to a significantly faster death rate than *P. aeruginosa* alone it could be inferred that this was due to *P. aeruginosa* becoming more virulent. Wax worms were injected with various concentrations of ethanol killed germlings and monitored for survival (Figure 3.22).

As no significant difference in survival rates was found between the different concentrations of dead Af293 8 h germlings used (Logrank test $p>0.05$), and very few worms died, it was decided that the highest concentration (1×10^6 cells/ml) should be used for combinational studies to increase the exposure of *P. aeruginosa* to Af293.

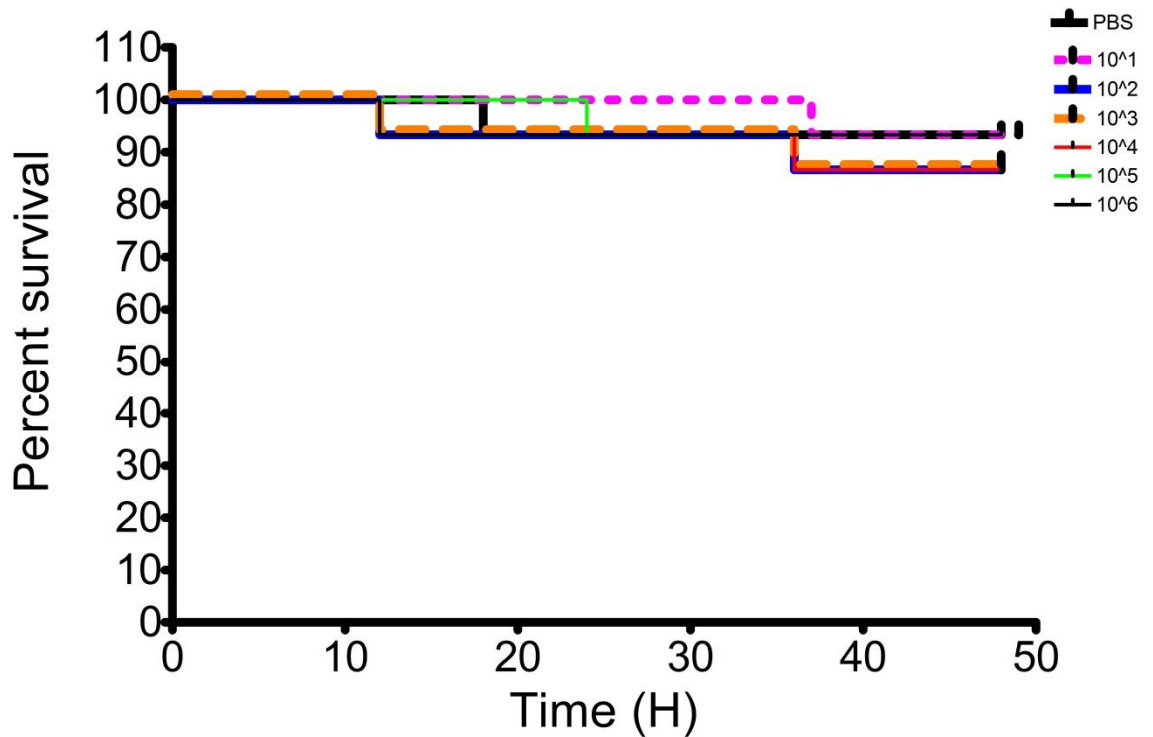


Figure 3.22. Survival curves of killed *Aspergillus fumigatus* germlings in the *Galleria mellonella* infection model.

Wax worm larvae were inoculated with 10 μ l of serial dilutions (10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10^1 germlings/ml) killed Af293 germlings suspended in PBS, as well as a suitable control. Survival was monitored for 48 h. There was no significant difference ($p>0.05$) in dose response. Kaplan-Meier survival curves were determined for three independent experiments each using five worms per condition. $n=3$.

3.6.3. *Survival rates of G. mellonella injected with P. aeruginosa in combination with killed A. fumigatus germlings*

To determine if the presence of dead Af293 contributed to PA14 causing death at a more rapid rate in comparison with PA14 alone, PA14 was combined with dead 8 h Af293 germlings in PBS before injection into the worms. Survival was then against suitable controls: PA14 alone, dead 8 h Af293 germlings alone and PBS alone (Figure 3.26).

Although both PA14 alone and PA14 with dead Af293 germlings were significantly (Logrank test $p < 0.001$) different than that of dead Af293 germlings alone, the median survival time for both was the same (18 h). It was shown that PA14 combined with dead Af293 germlings often caused greater morbidity than PA14 alone at most individual time points. For example, at 20 h 33% of wax worms injected with PA14 were still alive in comparison with 20% of wax worms injected with PA14 combined with dead Af293 germlings.

The pigmentation of worms, a sign of disease, also varied between the two conditions (Figure 3.23). For example, during one of the three repeats, although all worms were still alive 14 h post-injection, 4 worms demonstrated pigmentation when injected with a combination of PA14 and dead Af293 germlings in comparison to those injected with PA14 alone, of which only 1 worm showed signs of pigmentation (Figure 3.28). This could hint towards the dead Af293 germlings having some effect on the PA14, but it is inconclusive and the survival rate data shows no clear effect (Logrank Test $p > 0.05$). It was shown that the median time for onset of pigmentation in worms was 14 h for worms injected with combinations of PA14 and dead germlings in comparison with 16 h for PA14 alone. This was not shown to be a significant trend however using curve comparison.

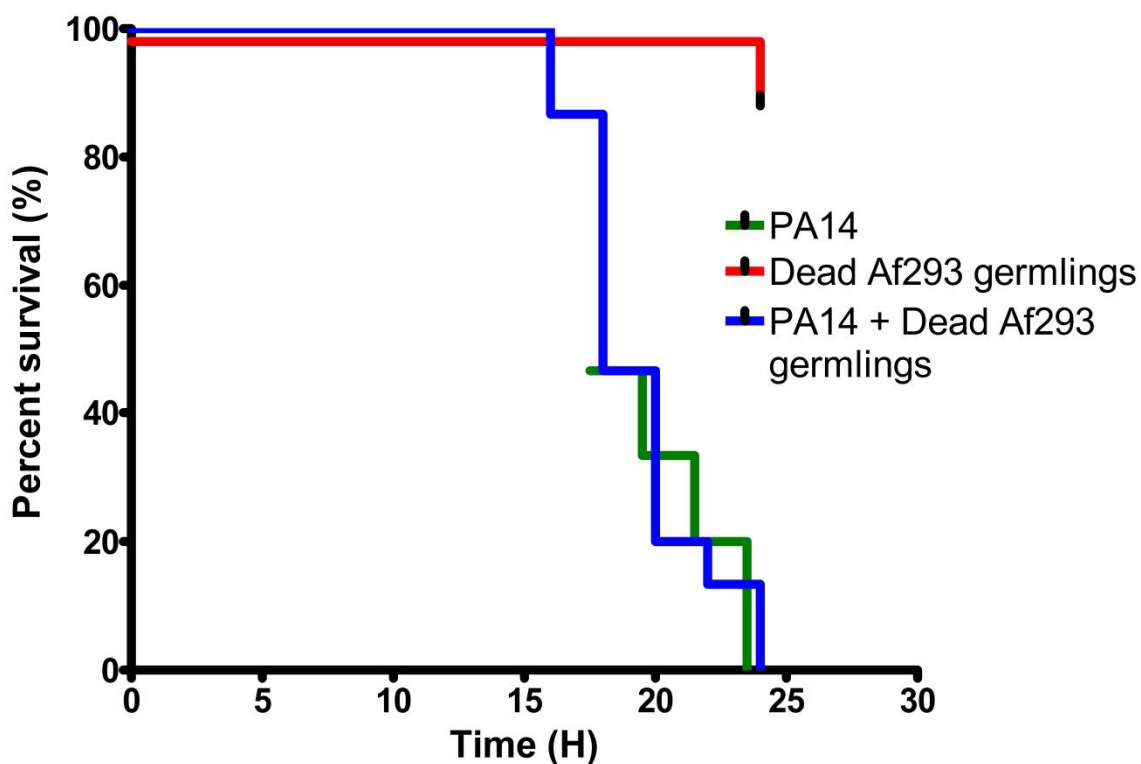


Figure 3.23. Effect of killed *Aspergillus fumigatus* germlings on *Pseudomonas aeruginosa* virulence in the *Galleria mellonella* infection model.

Wax worm larvae were inoculated with 10µl of PA14 at 1×10^3 cells/ml, killed 8 h Af293 germlings at 1×10^6 germlings/ml and a co-incubation of 1×10^3 cells/ml PA14 with 1×10^6 killed germlings/ml Af293. Survival was monitored for 24 h. Kaplan-Meier survival curves were determined for three independent experiments each using five worms per condition and showed a dose-dependent response. $n=3$.

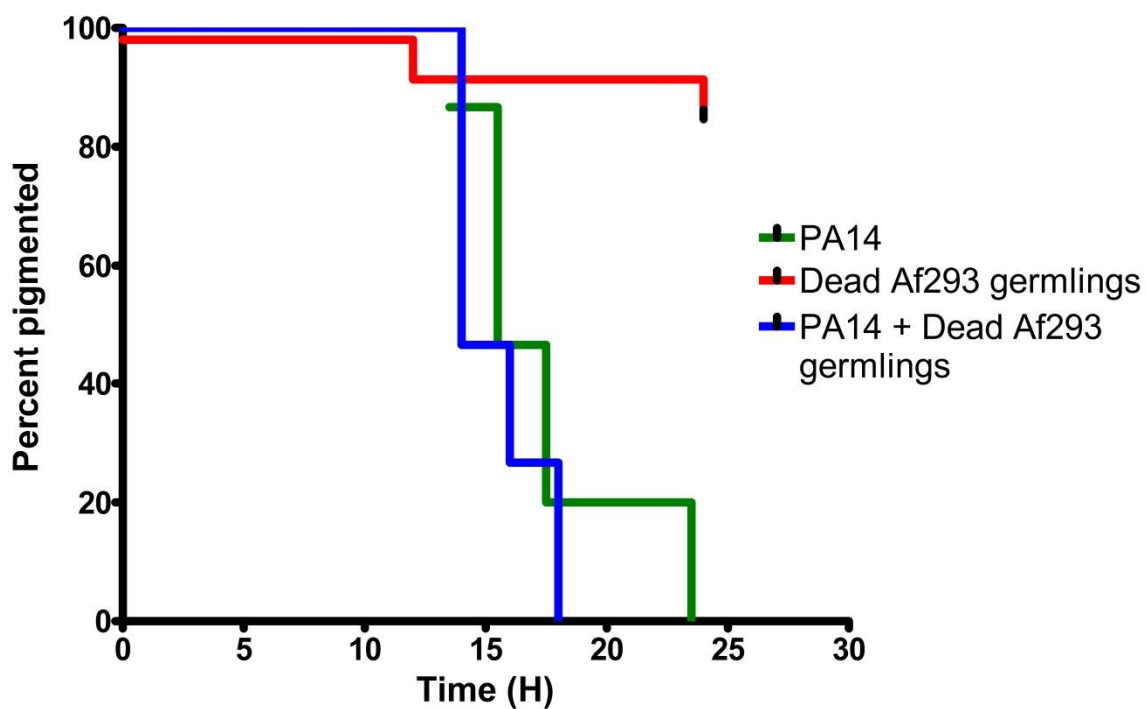


Figure 3.24. Effect of killed *Aspergillus fumigatus* germlings combined with *Pseudomonas aeruginosa* on pigmentation the *Galleria mellonella* infection model.

Wax worm larvae were inoculated with 10 μ l of PA14 at 1x10³ cells/ml, killed Af293 8 h germlings at 1x10⁶ germlings/ml and a co-incubation of 1x10³ cells/ml PA14 with 1x10⁶ killed germlings/ml Af293. Pigmentation was monitored for 24 h. Kaplan-Meier survival curves were determined for three independent experiments each using five worms per condition and showed a dose-dependent response. n=3.

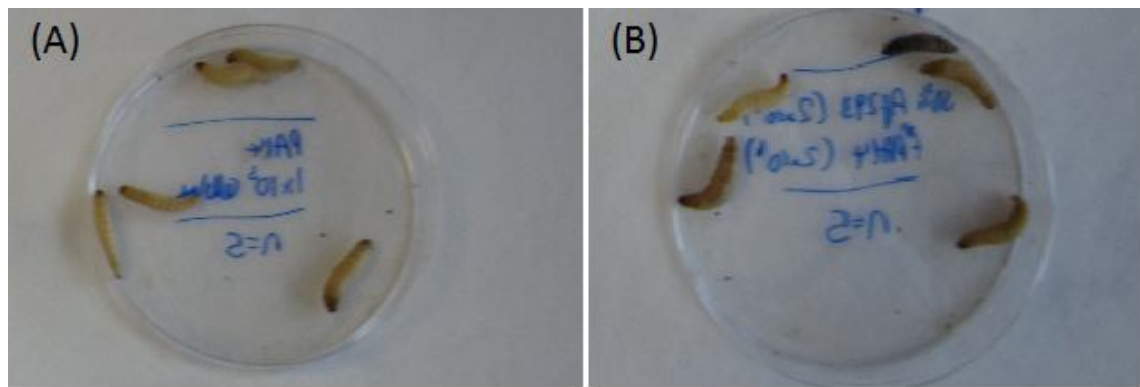


Figure 3.25. Increased pigmentation of wax worms 14 hours post-injection with *Pseudomonas aeruginosa* when combined with dead *Aspergillus fumigatus* germlings. Photographs of wax worms injected with (A) PA14 at 1×10^3 cells/ml and (B) PA14 at 1×10^3 cells/ml combined with dead Af293 germlings at 1×10^6 germlings/ml in PBS 14 h post-injection.

4. Discussion

Cystic Fibrosis (CF) is the most common lethal genetic disorder within the Caucasian population and leaves those affected vulnerable to chronic infection of the respiratory tract from a variety of microbial species, with *Pseudomonas aeruginosa* and *Aspergillus fumigatus* serving, respectively, as important bacterial and fungal species implicated (Rajan and Saiman 2002; Bakare *et al.*, 2003; Davies *et al.*, 2007). It has been shown that co-infection from both species within the same host occurs regularly, meaning that bacterial fungal interactions readily occur *in vivo*. How these organisms interact with one another may result in phenotypes that have detrimental effects on the host. Therefore, improving our understanding of how prokaryotes interact with eukaryotes within the context of the CF lung may help provide insight into improving clinical management (Leclair and Hogan 2010; Mowat *et al.*, 2010; Peleg *et al.*, 2010).

It had previously been shown that *P. aeruginosa* can inhibit the growth of *A. fumigatus*. Environmental strains of *Pseudomonas* in the soil were shown to antagonistically inhibit *A. flavus* up to 100-fold from samples taken from a Mississippi cornfield (Palumbo *et al.*, 2010). The inhibition of *A. fumigatus* by *Pseudomonas* has also been described in an investigation in which a strain of *P. fluorescens* isolated from cassava starch and flour was observed to cause moderate inhibition of the fungus *in vitro* using a plate streaking method (Osha 2005). In the context of the species interacting in CF patients, this inhibition possibly occurs through action of small secreted heat-stable molecules (e.g. decanol), pyocyanin and 1-hydroxyphenazine (Kerr *et al.*, 1999; Mowat *et al.*, 2010). This was demonstrated by showing that both filtered and heat-killed supernatants from *P. aeruginosa* caused the same inhibitory effect in *A. fumigatus* as untreated *P. aeruginosa* supernatants. A well plate assay by Kerr and co-workers in 1999 in which pyocyanin and 1-hydroxyphenazine were added to BHI agar in plastic wells containing fungal indicator strains at a 35mm radius showed that pyocyanin caused inhibition in both *C. albicans* and *A. fumigatus*. However, whilst dose-dependent inhibition of *A. fumigatus* occurred at higher levels (>19 µg/well) than in *C. albicans* (35 µg/well), complete inhibition of *A. fumigatus* growth was seen in comparison with partial inhibition of *C. albicans*.

The investigation by Ramage and colleagues in 2010 demonstrated a similar effect by showing that live *P. aeruginosa* incubated with live *A. fumigatus* conidia led to a significantly reduced fungal biomass (14.5%)(Mowat *et al.*, 2010). Despite this, *P. aeruginosa* was not shown to inhibit pre-formed *A. fumigatus* biofilm. This also correlates with the work of Ramage's group which showed an insignificant reduction in fungal biomass when *P. aeruginosa* cells were incubated with mature *A. fumigatus* biofilm (84.8% of control). The *P. aeruginosa* quorum sensing molecules decanol, decanoic acid and dodecanol were solubilized and used to treat both *A. fumigatus* conidia and mature biofilm at a variety of concentrations. These were implicated in inhibition of *A. fumigatus* by causing inhibition of fungal biomass (up to 80%) or disruption of fungal biofilm (up to 85%). To confirm this effect, a simple experiment was set up on RPMI-1640 agar plates. Filter discs impregnated with *P. aeruginosa* led to inhibition when grown on an *A. fumigatus* lawn, whereas *A. fumigatus* discs did not cause inhibition on a lawn of *P. aeruginosa* under the same conditions. This inhibition has previously been shown in *C. albicans* by growing a known antifungal strain of *P. aeruginosa* on a blood agar plate for 18-24 h aerobically before removal and chloroform killing of bacteria, and then demonstrating that *C. albicans* inoculated on this plate did not grow. The *P. aeruginosa* virulence factor pyocyanin was shown to cause a zone of inhibition in *C. albicans* growth in a well plate assay (Kerr 1999). This was the rationale for using pre-formed *A. fumigatus* biofilms (8, 12, 24 and 48 h biofilms) as opposed to co-incubating *P. aeruginosa* with uncultured 0 h *A. fumigatus* conidia.

With this in mind, the main aim of these studies was to determine whether the interactions between *P. aeruginosa* and pre-formed *A. fumigatus* biofilm led to an increased risk of morbidity and mortality in the host, with a surrogate *P. aeruginosa* virulence marker being selected as an *in vitro* method of detection. *P. aeruginosa* possesses a vast array of potent virulence factors for potential selection during these studies, including pyocyanin and phospholipase C (Cox 1986; Korbsrisate *et al.*, 2007). Elastase was selected as a suitable marker of *P. aeruginosa* virulence due to it being a well-documented and commonly expressed virulence factor, produced in over 75% of *P. aeruginosa* strains, and having a known pathogenic effect due to its cleavage of host elastin causing degradation of the lung structure (Kon *et al.*, 1999; Kuang *et al.*, 2011). The

availability of a developed biochemical assay which could be used to quantify the amounts of elastase produced efficiently (in micrograms per millilitre) and as it was a well-documented cause of morbidity in the host made it a suitable choice for these investigations (Kothary *et al.*, 1984; Caballero *et al.*, 2001; Hoge R. 2010).

The ECR assay was optimised for the *in vitro* quantification of elastase produced by *P. aeruginosa* in the presence of *A. fumigatus* biofilms. The main consideration for this assay on a practical level was avoiding ECR powder being pipetted from samples into the plate used for optical density readings, as this led to false positives at early stages of the project. A standard curve using known quantities of elastase was also run for each ECR plate during the study in order to more accurately quantify the levels of elastase present in the supernatants tested (Kothary *et al.*, 1984). In order to demonstrate that any changes in elastase produced by *P. aeruginosa* was not simply due to the presence of any physical contact; inorganic latex beads were selected as a control. It was shown that *P. aeruginosa* elastase production was not significantly different in the presence of an inorganic substrate. Results were standardised against any elastolytic activity occurring in an *A. fumigatus* only control, as it is known that *A. fumigatus* too can cause elastolytic activity (Kothary *et al.*, 1984; Rhodes *et al.*, 1988). For example, of 75 *A. fumigatus* strains screened for elastase by Kothary *et al.* in 1984, in liquid and solid medium containing elastin, 71 strains were shown to produce elastase in solid agar, and 33 demonstrated elastase production in liquid media (Kothary *et al.*, 1984). Moreover, Rhodes displayed via the use of Rose Bengal-elastin agar plates that all strains of *A. fumigatus* isolated from a sample of patients suffering from invasive aspergilliosis produced elastase. This ensured that only *P. aeruginosa* elastase was taken into account.

It is known that fungal biofilms can lead to the up-regulation of virulence factors in *P. aeruginosa*, as it has been shown, for example, that *Candida albicans* can stimulate phenazine production in dual-species biofilms (Cugini *et al.*, 2010). This was demonstrated by inoculating *P. aeruginosa* strain PA14 onto *C. albicans* (strain SC5314) lawns grown on agar plates and showing that observable zones of red pigmentation (indicative of phenazine) occurred due to this in comparison to

no red pigmentation being found for both species alone. Therefore, this served as a basis for testing if *A. fumigatus* exerted a similar effect on *P. aeruginosa*. Indeed, it was shown that the presence of dead *A. fumigatus* biofilm often resulted in a significantly higher level of elastase produced by *P. aeruginosa*, and this pattern was repeated over several strains (PA01, PA14, both clinical mucoid and non-mucoid strains PA 06.72747A and PA 06.72747AC) to varying degrees, and also within another key CF bacterial pathogen *Burkholderia cenocepacia* K56-2 (Drevinek and Mahenthiralingam 2010). One theory as to why the level of elastase increased in the presence of dead *A. fumigatus* biofilm is that the *P. aeruginosa* bacteria scavenge nutrients from the dead fungal biofilm, allowing for improved growth conditions in comparison to *P. aeruginosa* in media alone (Hogan. 2009). It is also possible that *P. aeruginosa* up-regulates *LasB*, the gene encoding for elastase, in response to encountering *A. fumigatus* (Rust *et al.*, 1996).

It was also shown that disruption of the dead *A. fumigatus* biofilm structure did not result in a significant increase in elastase, whereas the undisturbed dead biofilm caused a significant increase in elastase production by *P. aeruginosa*. It is possible that a specific interaction between *P. aeruginosa* and the fungal biofilm structure is essential to the mechanism resulting in enhanced elastase production. It is also possible that a factor released by *A. fumigatus* during the bead beating process into the supernatants, which would be contained within the fungal cells in the undisturbed biofilm, could counter-act the mechanism usually allowing *P. aeruginosa* to produce more elastase. Further work is required to discover the exact mechanism by which *P. aeruginosa* shows increased elastolytic activity in the presence of dead *A. fumigatus* biofilm, and why the structural integrity of the dead fungal biofilm appears to be key to this interaction. As an increase in elastase had not been observed when *P. aeruginosa* was exposed to live *A. fumigatus* biofilm, it was decided not to use a disrupted live biofilm in this study. However, this could be carried out in future work in order to explore in more detail the interaction between *A. fumigatus* biofilm and *P. aeruginosa* and its virulence factors.

Gene expression results for PA01 *LasB*, the gene, which encodes for elastase in *P. aeruginosa*, did not show a significant difference between PA01 alone and

PA01 grown with dead 24 h *A. fumigatus* biofilm at multiple time points post-exposure (1, 4, 8 and 24 h). This was in relation to the housekeeping gene *rpoD*, a sigma factor which has been indicated as a useful housekeeping gene within the literature (Potvin *et al.*, 2008). The lack of gene expression data to corroborate an increase in *LasB* activity could perhaps imply that another genetic pathway is utilised to cause the increase in elastolytic activity. The experiment should perhaps be repeated with a more robust housekeeping gene system. For example, the use of *rpoD* and *proC* (which encodes pyrroline-5-carboxylate reductase) simultaneously for housekeeping genes within qPCR studies has been recommended (Savli *et al.*, 2003). It is not necessary that elastase mRNA levels and elastase protein levels correlate, an increase in elastase mRNA may not be required to result in increased elastase activity. There could be increased transcription with increased translation matched by increased degradation of translated mRNA and no apparent change in mRNA levels when assessed by qPCR. It could simply be an increased efficiency in elastase mRNA translation into elastase, more efficient intracellular processing of elastase and secretion of elastase in *P. aeruginosa* given the right stimulus. Regardless, the *in vitro* ECR data did show a clear correlation between dead *A. fumigatus* and increased elastolytic activity in *P. aeruginosa*. This served as a basis for further investigation into how combining the two species impacted upon the health of the host, through cell culture studies and also within an insect model of disease.

In order to further demonstrate the validity of selecting elastase as the virulence factor of interest, the effect of various concentrations of elastase on a human lung epithelial cell line (A549) was analysed in terms of cell viability, using the Alamar Blue assay, and on morphological changes as seen by light microscopy (Lieber *et al.*, 1976; O'Brien *et al.*, 2000). It has been reported that elastase leads to a marked decrease in A549 cell viability as determined by the MTT assay (Nakajoh *et al.*, 2003). It was shown using the Alamar Blue assay in this study, however, that elastase did not lead to a loss in cell viability as expected. In fact, it was found to cause no significant difference in cell viability between concentrations of 0.5 and 16 µg/ml of elastase, and a significant increase in cell viability by 128 µg/ml. This surprising result could be explained through morphological evaluation of A549 cells, which appeared to be detached from the

plastic surface of the 24-well plate. Therefore, through nuisances in the experimental design, the data presented herein provided conflicting data sets, i.e. detached cells were either washed out of the cell, as described by Nakajoh and colleagues (2003), or they were left within the wells prior to metabolic quantification.

The ability of elastase to cleave elastin resulting in A549 cell detachment has been previously detailed within the literature (Van Wetering *et al.*, 1997). The cleaving of elastin, leading to respiratory cells being removed from the surface of the respiratory tract, would naturally lead to host damage (Bruce *et al.*, 1985). The discrepancy between the data collected in this study and that of Nakajoh *et al.*'s work from 2003 could be due to multiple factors, including the use of the MTT assay over Alamar Blue. Alamar Blue is an assay which does not require killing the cells in order to measure viability in comparison with MTT, and it has been shown previously that Alamar Blue is generally slightly more sensitive than MTT (O'Brien *et al.*, 2000; Hamid *et al.*, 2004). In order to further validate the effect of elastase on A549 cell viability, the lactate dehydrogenase (LDH) and neutral red assays could be utilised as alternative methodologies (Fotakis and Timbrell 2006). The neutral red assay indicates the number of living cells in culture as neutral red dye accumulates in the cytoplasm of living cells with absorbance being directly proportional to the number of living cells (Repetto *et al.*, 2008). The LDH assay, however, allows for a more direct measure of cell damage and death. As leakage of cellular enzymes such as LDH are known to occur during cell damage, and LDH catalyses the conversion of lactate and NADH to pyruvate and NAD, respectively. this reaction then stimulates conversion of iodotetrazolium by diaphorase to form a formazan dye which can be measured by an increase in fluorescence (Arechabala *et al.*, 1999).

The effect of supernatants collected from *P. aeruginosa*, *A. fumigatus* and combinational cultures of the two was gauged by treating A549 cells for 24 h before measuring cell viability via the Alamar Blue assay. One problem which arose from this approach was *Aspergillus* was so cytotoxic that the presence of live Af293 supernatants alone caused such a loss in cell viability that combinational studies were difficult to extract meaningful data. The cytotoxicity of *A. fumigatus* has been established in other cell lines such as

macrophages (Kamei *et al.*, 2002). An investigation into the cytotoxicity of clinical samples of *Aspergillus* isolated from hospitals utilising the MTT assay showed that 84% of tested strains were cytotoxic, and high cytotoxicity was demonstrated particularly in *A. fumigatus* (Gniadek *et al.*, 2011). A highly significant difference was found to occur between PA14 grown alone and PA14 grown with dead Af293, however. Interestingly, this showed that PA14 grown alone caused significantly more loss in viability of A549 cells in comparison with PA14 grown with dead Af293. This fits with the data from the cell viability assay carried out using varying concentrations of elastase. In that study, high levels of elastase increased cell detachment from the plate surface and also led to an increase in Alamar Blue reduction, as is the case for *P. aeruginosa* grown with dead fungal biofilm. Light microscopy of A549 cells treated with supernatants from PA14 alone and PA14 grown with dead *A. fumigatus* biofilm did show increased cell rounding, and detachment for PA14 grown with dead *A. fumigatus* biofilm supernatants, in comparison to PA14 alone. This potentially indicates increased elastolytic activity. As illuminating as *in vitro* experimental data can be, the full picture of how these interactions occur in nature requires a less simplistic model of infection, and this is where an *in vivo* model of infection becomes pertinent.

In order to analyse the effect of these interactions on a host system, the insect model *Galleria mellonella* was selected (Cotter *et al.*, 2000). There are numerous advantages to focussing on an insect model in favour of a mammalian system. The correlation between the immunity of insects and the immunity of mammals, as well as the high probability of conserved virulence mechanisms from pathogens across multiple host systems means that the insect model is indeed suitable for early experimental trials (Tan 2002). Moreover, they are easier to maintain in terms of living conditions and nutrition, and they allow the more complicated ethical issues of mammalian models to be avoided. Insect models also allow for higher throughput in terms of organisms tested and time taken for survival to be gauged. The costs are also kept low with these systems, and they are easier to handle (Scully and Bidochka 2006). It has been shown that pathogenicity of *A. fumigatus* in *G. mellonella* matches that of *A. fumigatus* in a mice model (Slater *et al.*, 2011).

A working model of how combinational infections with bacteria and fungi affected the survival of a host species existed within the literature (Figure 4.1) and was used as the basis of the wax worm studies within this project (Peleg *et al.*, 2010). Within this model, if the percentage survival for both species combined in a co-infection is equal to the survival rate of both species individually, it is considered an additive effect where both species could potentially be acting independently to cause host damage without any impact upon one another). If the host survival rate is lower than that of both species individually combined, then this implies a synergistic interaction in which one or both species cause an increase in virulence in the other, or one species creates conditions which the other exploits to cause increased host damage. If the host survival rate is higher than that of both species individually combined, then this could imply an antagonistic interaction in which one species removes the pathogenic effect of the other, or the host response to both species being there is stronger than for one species alone.

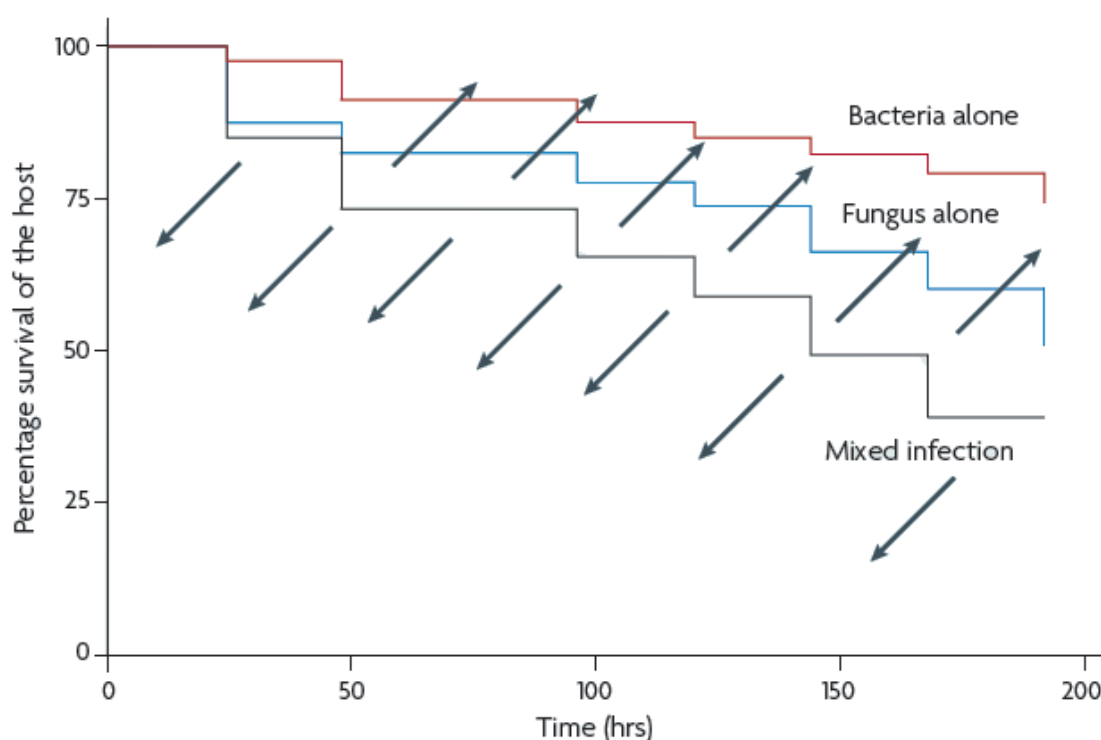


Figure 4.1. Theoretical survival curves for polymicrobial infections in an animal model.

Due to the complexity of bacterial-fungal interactions within a host, it is useful for a basic working model of how these interactions could impact upon the survival of the host, which can be applied to multiple experiments within this field. The percentage survival of the host species population is mapped against time (h). Arrows pointing upwards represent synergy in co-infections, and arrows pointing downwards represent antagonism in co-infections. Although these interactions are very complex, this basic diagram allows for a more unified interpretation of bacterial fungal interactions in host systems and how these relate to host survival (Peleg et al., 2010).

In contrast to this model, it was decided that killed 8 h *A. fumigatus* germlings would be combined with live *P. aeruginosa* as the results from the ECR assay had demonstrated that dead *Aspergillus* caused an increase in virulence in *Pseudomonas*. Moreover, the dead 8 h Af293 germlings had been shown to cause no significant impact on the survival rates of the worms. Therefore, the theory was that worms injected with PA14 combined with dead Af293 germlings would demonstrate a faster rate of killing in the worms in comparison to PA14 alone. The results from this study did not show a significant difference in survival rates of worms when PA14 was combined with killed germlings, however. Pigmentation rates of the worms was noted to be somewhat faster in those injected with both, but again this was not shown to be significantly different.

This could simply mean that although virulence is shown to increase *in vitro*, it did not do so *in vivo* within the conditions used. This could perhaps be due to the amount of PA14 being injected being below the necessary level for an increase in virulence. One possible explanation for this is that quorum sensing is required for the increased elastase production in *P. aeruginosa* exposed to dead Af293, and approximately one bacterium per injection is not a sufficient population density for this to occur (Rumbaugh *et al.*, 2000; Erickson *et al.*, 2002). It is also possible that there was an increase in the level of elastase produced, but given the lower population size it was not enough to manifest in a subsequent increase in morbidity. For example, no significant effect occurred in the A549 cell culture model until 128µg/ml. It is also possible, given the subjective nature of measuring infection in the wax worms, that there are changes within survival rates and morbidity in the worms, which are too subtle to be noticed within this experimental set-up. Focussing exclusively on PA01 may not have been the optimum approach for this study also, and other strains (e.g. PA14, PA 06.72747A) could have been utilised instead, including screening a panel of clinical isolates.

In terms of future work, it would be useful to determine the exact mechanism by which *P. aeruginosa* elastolytic activity increases in the presence of dead *A. fumigatus* biofilm, and likewise the mechanism by which it decreases in the presence of live *A. fumigatus* biofilm. A transposon mutant library could be utilised to this effect by using knockout mutants to determine which genes are essential to the mechanism. For example, work with quorum sensing knockout mutants ($\Delta LasI$ and $\Delta LasR$) already showed that the elastolytic activity was dependent on that quorum sensing pathway. Moreover, all work was carried out using the same strain of *A. fumigatus*, Af293, so the use of *A. fumigatus* mutant or clinical strains could also potentially reveal which genes within *A. fumigatus* are key to its effect on *P. aeruginosa* (both for live and dead *Aspergillus* biofilm). On a larger scale, using more bacterial species to test how universal this effect is could be useful, such as further *B. cenocepacia* strains, and other important CF pathogens such as *Staphylococcus aureus* and *Stenotrophomonas maltophilia* (Demko *et al.*, 1998; Goodrich *et al.*, 2009).

The wax worm model of infection could also be utilised with other strains of *P. aeruginosa*, or *P. aeruginosa* treated with *A. fumigatus* under other conditions e.g. *P. aeruginosa* grown with 24 h Af293 biofilm. The combinational effect of the two pathogens could also be examined by this method as per Figure 4.1, but finding an effective dosage for both species, which does not lead to rapid death could be problematic. Injecting the wax worms directly with elastase at various concentrations to establish it as a cause of death in the worms could also be a useful control. Monitoring the survival of worms more frequently could also be valuable.

Using alternative virulence factors to examine the effect of *A. fumigatus* on *P. aeruginosa* virulence could also be of interest, for example utilising a spot test on egg yolk agar in order to assay phospholipase C in *P. aeruginosa* from various conditions (Price *et al.*, 1982). A more ambitious approach towards exploring the adaptation of bacteria to *A. fumigatus* would be to use RNA microarrays or RNA-Seq to screen for gene regulation across the entire *P. aeruginosa* genome with a transcriptome approach, to detect which genes are affected and translate this into potential explanations for the increase in elastolytic activity (Palma *et al.*, 2003; Chang *et al.*, 2005; Waite *et al.*, 2005). RNA-Seq has been shown to be more sensitive, reproducible and to have a more dynamic range than microarrays, but it has also been suggested that there is an advantage to utilising both methods in a complimentary fashion (Kogenaru *et al.*, 2012).

Overall, this study hints at intriguing interactions between *P. aeruginosa* and *A. fumigatus*, with a surrogate virulence factor showing that bacterial fungal interactions can exert a clear influence on the activity of a bacterial virulence factor in a synergistic fashion. However, given the complexities of the CF lung ecology, in which multiple bacterial and fungal species, all of which can be present in multiple strains, come into contact with each another and express multiple virulence factors and behaviours as a result, much work remains to be done in order to utilise this knowledge. The ability to identify the mechanisms by which *P. aeruginosa* increases its virulence expression in response to *A. fumigatus* leaves us better enabled to interfere with this interaction through use of drugs and medication, and confer health benefits towards the CF patients still affected by these interactions.

In conclusion, the main findings of this investigation were as follows:

- I. *P. aeruginosa* elastase production is significantly increased within select strains in response to the presence of dead *A. fumigatus* biofilm. The structure of the fungal biofilm is key to this increase in elastase production.
- II. *P. aeruginosa* elastase production is significantly decreased within select strains in response to the presence of live *A. fumigatus* biofilm.
- III. *B. cenocepacia* elastase production is significantly decreased within the K56-2 strain in response to the presence of live *A. fumigatus* biofilm.
- IV. An inorganic substrate (in this case latex beads) does not cause an increase in elastase production, demonstrating that the presence of any physical scaffold in culture does not cause *P. aeruginosa* elastase production.
- V. A qPCR approach did not demonstrate a significant increase in LasB (which encodes for elastase) expression in PA01 treated with various stages of *A. fumigatus* biofilm.
- VI. Elastase causes the detachment of A549 cells from plastic surfaces *in vitro* but does not cause cytotoxicity as measured by the Alamar Blue assay.
- VII. A significant decrease in survival time was not noted in a *G. mellonella* model of infection when *P. aeruginosa* was co-incubated with dead *A. fumigatus* germlings.

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